Optimizing Foreign Gene Expression in Recombinant Fowl Adenovirus 9 Vectors

by

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ABSTRACT

Optimizing Foreign Gene Expression in Recombinant Fowl Adenovirus 9 Vectors

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Our laboratory focuses on the molecular characterization of a non-pathogenic strain of fowl adenovirus (FAdV) 9 and its development as a versatile vaccine vector platform. The objectives of this study were to optimize transgene expression by recombinant viruses. High expression promoters and a post-transcriptional regulatory element were evaluated for their ability to improve expression of enhanced green fluorescent protein (EGFP) in recombinant FAdVs. These findings were compared to our current system that employs the human cytomegalovirus (CMV) promoter to express a transgene. EGFP expression was assessed by fluorometry and Western blots. A synthetic CMV enhancer/chicken β-actin (CAG) promoter and the human elongation factor 1 alpha (EF1α) promoter significantly increased expression of EGFP compared to the CMV promoter. However, expression was significantly decreased in the presence of woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). The results provide novel insight into avian vaccine design and optimization of transgene expression by FAdV vectors.
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DECLARATION OF WORK PERFORMED

I declare that James Ackford performed all the work reported in this thesis, with the exceptions listed below, under the supervision of Drs. Éva Nagy and Peter Krell and an advisory committee member of Dr. Gregory Bédécarrats.

1) David Leishman performed protein quantification of recFAdV whole cell lysate by Bradford assay for the analysis of EGFP expression with Western immunoblot.
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LIST OF ABBREVIATIONS

AdV       adenovirus
AI        avian influenza
β-actin   chicken β-actin promoter
BAdV      bovine adenovirus
bGH       bovin growth hormone
BHV       bovine herpesvirus
bp        base pair
CAdV      canine adenovirus
CAG       CMV enhancer/chicken β-actin promoter
CAR       coxsackie and adenovirus receptor
CAT       chloramphenicol acetyltransferase
CAV       chicken anaemia virus
CD        cluster of differentiation molecule
ChAdV     chimpanzee adenovirus
CEL       chicken embryo fibroblast cells
CELi      primary chicken embryo liver cells
CH-SAH    chicken liver hepatoma cells
CMV       cytomegalovirus promoter
CPE       cytopathic effect
DAdV      duck adenovirus
DF-1       chicken embryo fibroblast cells
DMEM-F12  Dulbecco’s modified Eagle’s medium and nutrient mixture Ham’s F-12 medium
DSG2      desmoglein-2
E. coli   Escherichia coli bacteria
EDS       egg drop syndrome
EF1α      human elongation factor 1 alpha promoter
EGFP      enhanced green fluorescent protein
ELISA     enzyme-linked immunosorbent assay
FAdmid    infectious FAdV-9 DNA plasmid
FAdV      fowl adenovirus
FBS       fetal bovine serum
FPV       fowlpox virus
F/R       ratio of fluorescence over luminescence
GC        guanine-cytosine
GFP       green fluorescent protein
GoAdV     goose adenovirus
GUSB      β glucuronidase
HA  hemagglutinin
HAdV  human adenovirus
HE  hemorrhagic enteritis
hGH  human growth hormone
HIV  human immunodeficiency virus
HN  hemagglutinin-neuraminidase
h.p.i.  hours post-infection
h.p.t.  hours post-transfection
HPS  hydropericardium syndrome
HRP  horseradish peroxidase
HSPGs  heparin sulfate proteoglycans
HSV  herpes simplex virus
IBDV  infectious bursal disease virus
IBH  inclusion body hepatitis
IBV  infectious bronchitis virus
Ig  immunoglobulin
ITR  inverted terminal repeats
kb  kilo base pair
kDa  kilodalton
L2R  fowlpox virus early/late promoter
LB  Luria-Bertani
MAdV  murine adenovirus
MHC  major histocompatibility complex
MLP  major late promoter
MOI  multiplicity of infection
NDV  Newcastle disease virus
NeoR  neomycin resistance
NHAdV  non-human adenovirus
nt  nucleotide
OAdV  ovine adenovirus
ORF  open reading frame
PAdV  porcine adenovirus
PBS  phosphate buffered saline
PCR  polymerase chain reaction
Pfu  plaque forming units
pHMR  intermediate construct for recombination with pFAdV-9Δ4
PiAdV  pigeon adenovirus
PsAdV  psittacine adenovirus
pTP  terminal protein precursor
QT-35  quail fibroblast cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAAV</td>
<td>recombinant adeno-associated virus</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEAP</td>
<td>secreted alkaline phosphate</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen free</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TAdV</td>
<td>turkey adenovirus</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris buffered saline supplemented with 0.1% Tween 20</td>
</tr>
<tr>
<td>TGEV</td>
<td>transmissible gastroenteritis virus</td>
</tr>
<tr>
<td>TP</td>
<td>terminal protein</td>
</tr>
<tr>
<td>TR</td>
<td>tandem repeat</td>
</tr>
<tr>
<td>TSAdV</td>
<td>tree shrew adenovirus</td>
</tr>
<tr>
<td>UBC</td>
<td>ubiquitin C</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VF</td>
<td>viral flanking region</td>
</tr>
<tr>
<td>VV</td>
<td>vaccinia virus</td>
</tr>
<tr>
<td>WPRE</td>
<td>woodchuck hepatitis virus post-transcriptional regulatory element</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
<tr>
<td>Δfold</td>
<td>fold change in activity</td>
</tr>
</tbody>
</table>
Chapter 1. Literature Review

Adenoviruses (AdV) are large double-stranded DNA viruses that have been isolated from a wide variety of vertebrates. These viruses are extensively studied for their use as vaccine and gene therapy vectors, and to date, adenoviral vectors have been used globally in 23.5-26% of all clinical trials (Crystal, 2014; Lopez-Gordo et al., 2014). This is due to the fact that they present many advantages over other viral vectors, as they are safe, stable, grow to high titers, can accommodate large inserts of foreign DNA, are easy to manipulate, induce a strong innate and adaptive immune responses, and lastly are able to transduce a wide variety of cell types and drive transgene expression (Lopez-Gordo et al., 2014).

Fowl adenoviruses (FAdV), members of the genus Aviadenovirus, are ubiquitous amongst poultry and demonstrate various levels of pathogenicity (Hess, 2013). Although FAdVs have not been extensively studied, the current research has focused on developing these viruses as vector system for poultry and mammals, with particular interest in serotypes 8 and 9 (Ojkić and Nagy, 2001; Johnson et al., 2003; Corredor and Nagy, 2010b; Greenall et al., 2010). In our laboratory, a system to generate recombinant FAdV-9 viruses has been developed by exploiting the non-essential regions of genomic DNA and manipulating them in bacteria, referred to as the FAdmid system. FAdV-9 has shown to be an excellent vaccine vector since the engineered viruses demonstrate wild-type (wt) growth characteristics in vitro, yet shed decreased levels of virus in feces and induce a low immune response to the FAdV backbone, all while expressing foreign transgenes to stimulate the host immune system (Ojkić and Nagy, 2001; Corredor and Nagy, 2010a,b; 2011). While initial studies of these vectors have shown promise, additional research into vector design and gene expression is required in order to create more efficient recombinant viruses with higher levels of transgene expression.
1.1 Adenovirus biology

1.1.1 Adenovirus taxonomy

Adenovirus taxonomy is based on the organization of the viral genome, replication strategy, host range, and structure. Belonging to the family Adenoviridae, AdVs are grouped into five genera: Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus, and Ichtadenovirus (http://www.ictvonline.org/virusTaxonomy.asp). Further, members of each genus are organized into at least one or more species (Figure 1.1). Of the five genera, Mastadenovirus and Aviadenovirus are the two largest clades. To date, AdVs have been isolated from only vertebrates.

The genus Mastadenovirus includes viruses that infect only mammals, such as human adenoviruses (HAdVs) which are the most studied. Over sixty serotypes of HAdVs have been distinguished and are classified into several species (Human mastadenovirus A to Human mastadenovirus G). Human mastadenovirus C, which includes serotypes 2 and 5, are the most characterized AdVs at the molecular level. Other species within the genus Mastadenovirus include; Bat mastadenovirus A and Bat mastadenovirus B, Bovine mastadenovirus A to Bovine mastadenovirus C, Canine mastadenovirus A, Equine mastadenovirus A and Equine mastadenovirus B, Murine mastadenovirus A to Murine mastadenovirus C, Ovine mastadenovirus A and Ovine mastadenovirus B, Porcine mastadenovirus A to Porcine mastadenovirus C, Simian mastadenovirus A, and lastly Tree shrew mastadenovirus A (http://www.ictvonline.org/virusTaxonomy.asp).

The genus Aviadenovirus is distinct from the other AdV genera, as they infect only birds. The genus is organized into five fowl adenovirus (FAvD) species (Fowl aviadenovirus A to Fowl aviadenovirus E), Falcon aviadenovirus A, Goose aviadenovirus A, and
Figure 1.1. Phylogenetic analysis of the family Adenoviridae. Phylogenetic tree of adenoviruses constructed based on a distance matrix analysis of hexon amino acid sequences. Viruses, belonging to the family Adenoviridae, can be classified into five genera: Mastadenovirus (yellow background), Aviadenovirus (green background), Atadenovirus (blue background), Siadenovirus (orange background), and Ichtdenovirus (red background). Figure taken from Harrach et al., 2012.
Turkey aviadenovirus B (Table 1.1). Additionally, five new species have been proposed, which include: Duck adenovirus (DAdV-2), Meyer’s parrot adenovirus 1, Pigeon adenovirus (PiAdV-1), and psittacine adenovirus (PsAdV-1). The FAdV species are classified according to restriction fragment length polymorphisms (RFLP) of genomic DNA given by BamHI and HindIII (Zsák and Kisary, 1984), in addition to phylogenetic distance, host range, and pathogenicity. FAdVs can be grouped into 12 serotypes (FAdV-1 to 8a and 8b to 11) based on cross-neutralization test (Hess, 2000).

Members of the genus Atadenovirus infect a broad range of hosts including mammals, birds, reptiles, and marsupials. The genomes of these viruses are rich in adenine and thymine, and their genomic organization differs from those of the Mastadenovirus and Aviadenovirus genera. The genus is organized into five species: Bovine atadenovirus D, Duck atadenovirus A, Ovine atadenovirus D, Possum atadenovirus A, and Snake atadenovirus A. Viruses within the genus Siadenovirus have been isolated from both reptile and bird species. This genus is named as its members encode a putative sialidase gene in the left end of their genome (Davison et al., 2003). The genus is organized into five species: Frog siadenovirus A, Great tit siadenovirus A, Raptor siadenovirus A, Skua siadenovirus A, and Turkey siadenovirus A. Ichtadenovirus is the most recent genus accepted into the Adenoviridae family. This genus comprises a single species, Sturgeon ichtadenovirus A, found in white sturgeon (Kovács et al., 2003).
**Table 1.1.** Species and viruses within the genus *Aviadenovirus* from the International Committee on Taxonomy of Viruses (ICTV)

<table>
<thead>
<tr>
<th>Species</th>
<th>Serotype</th>
<th>Abbreviation</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fowl aviadenovirus A</em></td>
<td>Fowl adenovirus 1 (CELO)</td>
<td>FAdV-1</td>
<td>U46933</td>
</tr>
<tr>
<td><em>Fowl aviadenovirus B</em></td>
<td>Fowl adenovirus 5</td>
<td>FAdV-5</td>
<td>NC_021221</td>
</tr>
<tr>
<td><em>Fowl aviadenovirus C</em></td>
<td>Fowl adenovirus 4</td>
<td>FAdV-4</td>
<td>GU188428</td>
</tr>
<tr>
<td></td>
<td>Fowl adenovirus 10</td>
<td>FAdV-10</td>
<td>AF160185</td>
</tr>
<tr>
<td><em>Fowl aviadenovirus D</em></td>
<td>Fowl adenovirus 2</td>
<td>FAdV-2</td>
<td>AF339915</td>
</tr>
<tr>
<td></td>
<td>Fowl adenovirus 3</td>
<td>FAdV-3</td>
<td>AF508949</td>
</tr>
<tr>
<td></td>
<td>Fowl adenovirus 9</td>
<td>FAdV-9</td>
<td>AF083975</td>
</tr>
<tr>
<td></td>
<td>Fowl adenovirus 11</td>
<td>FAdV-11</td>
<td>KM231537</td>
</tr>
<tr>
<td><em>Fowl aviadenovirus E</em></td>
<td>Fowl adenovirus 6</td>
<td>FAdV-6</td>
<td>AF508954</td>
</tr>
<tr>
<td></td>
<td>Fowl adenovirus 7</td>
<td>FAdV-7</td>
<td>AF508955</td>
</tr>
<tr>
<td></td>
<td>Fowl adenovirus 8a</td>
<td>FAdV-8a</td>
<td>AF155911</td>
</tr>
<tr>
<td></td>
<td>Fowl adenovirus 8b</td>
<td>FAdV-8b</td>
<td>AF508958</td>
</tr>
<tr>
<td><em>Falcon aviadenovirus A</em></td>
<td>Falcon adenovirus 1</td>
<td>FaAdV-1</td>
<td>AY683541</td>
</tr>
<tr>
<td><em>Goose aviadenovirus A</em></td>
<td>Goose adenovirus 1</td>
<td>GoAdV-1</td>
<td>-</td>
</tr>
<tr>
<td><em>Turkey aviadenovirus B</em></td>
<td>Turkey adenovirus 1</td>
<td>TAdV-1</td>
<td>NC_014564</td>
</tr>
</tbody>
</table>
1.1.2 Adenovirus structure

Adenoviruses are non-enveloped viruses with icosahedral symmetry that range from 70-90 nm in diameter, whose structure has been confirmed by microscopy (Reddy et al., 2010; Berk, 2013) (Figure 1.2). The viral capsid is composed of 252 subunits, specifically 240 hexons and 12 pentons (Harrach et al., 2012). Other viral proteins include the cement and structural proteins (II, III, IIIa, IV, VI, VIII, and XI) and the viral core proteins (TP, V, VII, and mu) that directly interact with the viral DNA (Vellinga et al., 2005; Lopez-Gordo et al., 2014; Reddy and Nemerow, 2014). At each penton, a trimeric fiber protein protrudes from the capsid that has a high affinity for the coxsackie and adenovirus receptor (CAR) (Tomko et al., 1997). Further, due to variation in capsid protein lengths, composition, and sequence, binding affinity for other host cell factors has been reported, thus accounting for the wide cellular tropism of AdVs. These factors include: CAR, cluster of differentiation molecule (CD) 46, desmoglein-2 (DSG2), CD80, CD86, vascular cell adhesion molecule-1 (VCAM-1), heparan sulfate proteoglycans (HSPGs), major histocompatibility complex (MHC) class I-a2, sialic acid, dipalmitoyl-phosphatidylcholine, and lactoferrin (Sharma et al., 2009; Arnberg, 2012; Lopez-Gordo et al., 2014). While most AdVs present a single fiber protein at each penton, HAdV-40 and HAdV-41, like FAdVs, display two fiber proteins at their pentons that are encoded by a single gene (Pieniazek et al., 1990; Kidd et al., 1993; Chiocca et al., 1996). However, FAdV-1, FAdV-4, and FAdV-10 encode an additional fiber gene of nearly equal length (Chiocca et al., 1996; Griffin and Nagy, 2011; Marek et al., 2012).
**Figure 1.2. Adenovirus structure.** The icosahedral structure of a HAdV-2 virion (left), composed of major capsid and core proteins (middle). An electron micrograph of FAdV-9 (right) showing the characteristic double fibers (red arrow) of FAdVs. The black bar represents 100 nm. Figure taken and modified from Harrach *et al.*, 2012.
1.1.3 Genome organization

The adenovirus genome comprises a linear double-stranded DNA ranging from 26-48 kilobase (kb) in size, containing inverted terminal repeats (ITR) (Harrach et al., 2012). A virus encoded terminal protein (TP) is covalently linked to the 5’ end of each DNA strand. The central part of the genome encodes structural proteins that are well conserved throughout the viral family. It is speculated that this region is inherited from a common ancestor, as these genes are involved in the replication and packaging of viral DNA, and the formation of the virion. The left and right ends of the genome show large amounts of variation among genera, both in length and genetic content. These regions are considered genus-specific as they are involved mainly in virus-host interactions, such as counteracting the host immune responses, promoting cell cycle progression and inhibiting and/or promoting apoptosis, suggesting that they are designed for specific interactions with their respective hosts (Davison et al., 2003). Based on transcriptional analysis of the adenovirus genomes there can be up to four early-transcribed regions (E1-E4), two delayed early regions (IX and IVa2), and five late transcribed regions (L1-L5), which translate over 40 proteins from both strands of DNA through complex splicing (Davison et al., 2003).

1.2 Avian adenoviruses

Adenoviruses are ubiquitous pathogens infecting both commercial and wild birds globally. Viruses belonging to the genera Aviadenovirus, Siadenovirus, and Atadenovirus have all been reported to affect various bird species, however most of the viruses isolated from chickens, turkeys, and geese belong to the genus Aviadenovirus (Hess, 2013). The most notable diseases caused by avian AdVs outside of the genus Aviadenovirus, include hemorrhagic enteritis (HE) caused by HE virus a member of the genus Siadenovirus, and egg drop syndrome
(EDS) caused by DAdV-1 a member of the genus *Atadenovirus* (Hess, 2013).

### 1.2.1 Molecular biology of aviadenoviruses

Compared to mammalian adenoviruses, the molecular biology of avian adenoviruses is far less studied. The genomes of aviadenoviruses are considerably larger than those of mastadenoviruses, by approximately 20-45% (Harrach *et al.*, 2012). Complete nucleotide (nt) sequences and transcriptional maps have been determined for the genomes of FAdV-1 (Chiocca *et al.*, 1996; Payet *et al.*, 1998), FAdV-4 (Griffin and Nagy, 2011), FAdV-5 (Marek *et al.*, 2013), FAdV-8 (Grgić *et al.*, 2011), FAdV-9 (Ojkić and Nagy, 2000; 2001), FAdV-11 (Zhao *et al.*, 2015), DAdV-2 (Marek *et al.*, 2014b), TAdV-1 (Kajan *et al.*, 2010), TAdV-4 and TAdV-5 (Marek *et al.*, 2014a), PiAdV-1 (Marek *et al.*, 2014b), and goose adenovirus 4 (GoAdV-4) (Kajan *et al.*, 2012). However, most molecular studies have focused on FAdV-1 and FAdV-9. The genomes are 43,804 base pair (bp) and 45,063 bp in size, respectively. FAdV-5 has a genome size of 45,810 bp and is the largest FAdV genome determined to date (Marek *et al.*, 2013).

The genomic organization of aviadenoviruses is unique compared to members of the other AdV genera (Figure 1.3). Their genomes lack sequences homologous to the mammalian E1, E3, and E4 regions, yet E2 regions, delayed-early protein IVa2, and late proteins appear to be well conserved (Chiocca *et al.*, 1996; Ojkić *et al.*, 2002, Davison *et al.*, 2003). The left and right ends of the avian adenovirus genomes are termed E1 and E4, respectively, despite the lack of genetic similarity in these regions to other genera (Davison *et al.*, 2003). The left end open reading frames (ORFs) (E1 region) are located upstream of the IVa2 gene while ORFs at the right end (E4 region) map downstream of the fiber gene (Figure 1.3). The E1 and E4 regions of the genome show the most variation between avian adenovirus species, however the biological
**Mastadenovirus: HAdV-5 (Human mastadenovirus C) - 35,938 bp**

**Aviadenovirus: FAdV-9 (Fowl aviadenovirus D) - 45,063 bp**

**Figure 1.3. Genomic organization of mastadenoviruses and aviadenoviruses.** A comparison of the genomic organization of a mastadenovirus (HAdV-5) and aviadenovirus (FAdV-9) type species. The full genomes are depicted by solid black lines, with each tick mark representing 10 kb. Regions highlighted in orange represent genus-specific genes, while regions highlighted in white are conserved regions between the genera. Avian adenoviruses lack homologs to mammalian E1, E3, and E4 regions. It is suggested that genus-specific genes play a major role in modulating the host immune response, while the conserved genes are responsible for viral replication and assembly.
relevance of these differences is unknown (Marek et al., 2013).

The ITRs of aviadenoviruses vary in length from 39 bp (GoAdV-4) to 721 bp (DAdV-2) (Marek et al., 2014a). Generally, ITRs are conserved within a genome, however a nucleotide mismatch or a C-T transition can occur, such as seen in TAdV-1 and PiAdV-1. Comparisons between FAdV and HAdV ITRs show that the functional domain A (0-18 bp) is conserved, but there is less conservation among the B (19-39 bp) and C (40-51 bp) domains (Cao et al., 1998).

The left end of aviadenoviruses consists of six rightward oriented ORFs: ORFs 0, 1, 1A, 1B, 1C, and 2, which make up the E1 region, and three leftward oriented ORFs: ORFs 14, 13, and 12, that make up the E2 region (Kajan et al., 2012). ORFs 0 and 1A are part of the 5’ untranslated region (UTR) of ORFs 1 and 1B, respectively (Ojkić et al., 2002). ORFs 1 and 2 have homologies to the dUTPase and paroviral Rep protein, respectively (Cao et al., 1998; Ojkić et al., 2002). ORF1C of FAdV-9 has 36.8% identity to the bovine papillomavirus 1 E5 protein (Ojkić and Nagy, 2000). The leftward oriented ORFs 24, 14, 13, and 12 encode the viral DNA polymerase and the terminal protein precursor (pTP) (Ojkić et al., 2002). These ORFs are related to the ORF2 family and have been classified within the NS-1 family, superfamily III helicases (Washietl and Eisenhaber, 2003). The left end ORFs are conserved amongst all sequenced avian adenoviruses genomes with a few exceptions. GoAdV-4 lacks ORFs 0, 1B, and 13, but has unique ORFs 51 and 52 that lack detectable orthologues in other avian adenoviruses (Kajan et al., 2012). TAdV-1, FAdV-4, FAdV-8, and FAdV-9 all contain the additional ORF 14A, but only FAdV-4 contains additional ORFs 14B and 14C (Griffin and Nagy, 2011; Marek et al., 2013; Kajan et al., 2012). PiAdV-1 lacks ORFs 0, 1A, B, and C, however contains ORF 52 (Marek et al., 2014b).
The central region of avian adenoviruses, from IVa2 to the fiber gene, is universally conserved. Six late transcription regions (L1-L6) are identified in FAdV genomes (Ojkić and Nagy, 2002). In general, the expression of late aviadenovirus mRNA involves complex differential splicing and polyadenylation that resembles the transcriptional processing of mammalian adenoviruses. However, late transcripts from FAdVs have a bi-partite leader composed of two exons, rather than a tri-partite leader like mastadenoviruses. This feature appears to be a common characteristic among FAdVs (Payet et al., 1998; Sheppard et al., 1998b). Most of the late ORFs are encoded by a single species of transcript in FAdVs, however there are multiple transcripts, utilizing different distal splice acceptor sites, specific for proteins III, VII, X, 100k, and fiber (Ojkić and Nagy, 2002). Compared to other adenoviruses this is a unique feature, but the functional consequences of having multiple transcripts for these proteins are unknown (Lawrence, 1980; Payet et al., 1998; Ojkić and Nagy, 2002).

Contrary to most adenoviruses, FAdVs have two fibers protruding from each vertex in their capsid, which are thought to play an important role in infectivity and pathogenicity (Pallister et al., 1996; Schachner et al., 2014). Members of the species Fowl aviadenovirus A and Fowl aviadenovirus C encode two fiber genes, while members of the remaining species possess only a single fiber gene (Chiocca et al., 1996; Ojkić and Nagy, 2000; 2001; Griffin and Nagy, 2011; Grgić et al., 2011; Marek et al., 2012; 2013). The presence of two fibers in FAdV-1 may be responsible for its hemagglutinating properties, and efficient replication in embryonated eggs (Hess, 2013). TAdV-1, TAdV-5, GoAdV-4, and PiAdV-1 have two fiber genes, while TAdV-4 and DAdV-2 have a single gene (Marek et al., 2014b).

In aviadenoviruses, the E4 region is substantially larger compared to other adenoviruses, comprising of ORFs 22, 20A, 20, 19, 8, and 17. In FAdV-9, the right end ORFs are further
classified into three regions based on transcriptional analysis: E4, E5 and E6 (Ojkić et al., 2002). ORF19 encodes a putative triglyceride lipase that is most likely important during infection of birds, as lipase homologues are also found in viruses such as Marek’s disease virus (Washietl and Eisenhaber, 2003). ORF 8 encodes Gam-1, which has BCL-2 like properties for inhibition of apoptosis (Chiocca et al., 1996). Similar to the left end, the right end ORFs are well conserved amongst all sequenced avian adenoviruses, with a few exceptions. GoAdV-4 encodes additional ORFs 56, 55, 54, 19B, and 53 (Kajan et al., 2012). FAdV-1 encodes additional ORFs 9, 10, 11, and 26 (Chiocca et al., 1996; Payet et al., 1998). FAdV-4 encodes additional ORFs 4, 43, 16, and 19A (Griffin and Nagy, 2011; Marek et al., 2012). Both FAdV-8 and FAdV-9 encode the additional ORFs 11, 23, and 25 (Ojkić and Nagy, 2000; 2001; Grgić et al., 2011). ORFs 9, 10, 11, 20A, 23 and 25 encode putative membrane glycoproteins (Davison et al., 2003). FAdV-1 ORFs 9, 10, and 11 encode three putative type-I transmembrane glycoproteins that have immunoglobulin (Ig) like domains which may have equivalent function to mastadenovirus E3 19 kilodalton (kDa) protein in impairing MHC-I molecules from transport to the cell surface (Le Goff et al., 2005). Unique to other avian adenoviruses, PiAdV-1 encodes six novel ORFs in its right end, ORF 58, a paralogue of ORF 58A, and ORFs 59 – 62 (Marek et al., 2014b). Additionally, DAdV-2 contains a novel paralogue of ORF 55 named ORF 55A, and five additional novel ORFs (ORF 63-67) (Marek et al., 2014b).

Two tandem repeats (TR) are present at the right end of the FAdV-4, FAdV-5, FAdV-8, FAdV-9, and FAdV-11 genomes (Ojkić and Nagy, 2000; Marek et al., 2013; Griffin and Nagy, 2011, Zhao et al., 2015). The TR-1 region in FAdV-9 consists of five direct repeats that are 33 bp long each. The TR-2 region consists of thirteen direct repeats that are 135 bp long each, and is dispensable for virus replication in vitro and in vivo (Ojkić and Nagy, 2000). In FAdV-4, FAdV-
5, FAdV-8, FAdV-9, and FAdV-11, the TRs occur in the same place after ORF 8.

1.2.2 Diseases caused by avian adenoviruses

FAdVs are ubiquitous amongst commercial and wild poultry, and have been isolated and recovered from chickens, turkeys, pigeons, budgerigars, and mallard ducks (Hess, 2013). Many of these viruses are considered non-pathogenic, replicating in birds with little to no clinical signs of infection. However, some FAdVs have been associated with a number of diseases such as inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), gizzard erosion, proventriculitis, and tenosynovitis (Mazaheri et al., 1998; Domanska-Blicharz et al., 2011; Hess, 2013). The most notable diseases associated with FAdV infection are IBH and HPS. Important to disease spread, FAdVs are transmitted both vertically and horizontally, with virus being present in all excretions, including feces, tracheal and nasal mucosa, and also the semen (Grgić et al., 2006; Hess, 2013).

Inclusion body hepatitis is a disease usually seen in meat-producing birds between 3-7 weeks of age, with mortality ranges between 5-10%, and upwards of 30%. The liver is the primary organ affected, and is characterized by a pale, swollen, and friable appearance, in addition to petechial or ecchymotic hemorrhages (Hess, 2013). Co-infection with other immunosuppressive agents including infectious bursal disease virus (IBDV) and chicken anemia virus (CAV) is suggested to be a major predisposing factor in the development of IBH, although IBH has been reported as a primary disease (Reece et al., 1986). To date, all 12 serotypes of FAdV have been associated with IBH in broiler chickens (Hess, 2013). Specifically, serotypes FAdV-2, -6, -7, -8, and -11 are commonly associated with IBH. However in Canada FAdVs associated with IBH outbreaks have been genetically related to FAdV-2, -8a, and -11 (Ojkić et al., 2008a,b; Hess, 2013).
Hydropericardium syndrome, like IBH, primarily affects meat-producing birds between 3-6 weeks of age, and differs from IBH in that the mortality and incidence rate of HPS are much higher, between 20-80% (Hess, 2013). The disease is characterized by the accumulation of clear fluid in the pericardium, pulmonary edema, swollen liver, and pale swollen kidneys, and is primarily associated with FAdV-4 (Kim et al., 2008; Schachner et al., 2014). The variability in disease association amongst FAdVs makes it hard to assess their global economic importance, however clinical cases of IBH and HPS can be tracked worldwide (Ojkić et al., 2008a; Choi et al., 2012; Kajan et al., 2013; Mittal et al., 2014; Zhao et al., 2015).

1.3 Applications of adenoviruses

Adenoviruses have been applied as both gene therapy vectors and vaccine vectors due to their ability to infect a wide variety of cell types and tissues in both dividing and non-dividing cells (Crystal, 2014). In addition, AdV vectors can be propagated to high titers making them practical to employ \emph{in vivo}, and viral genomes remain as episomes in the nucleus of transduced cells, eliminating the concern of insertional mutagenesis due to random integration of the viral genome within the host-cell genome. Moreover, AdV vectors induce strong immunity when administered via parenteral or mucosal routes, the latter often desired as most infections occur at mucosal surfaces. Currently, adenovirus gene therapy vectors are applied in 23.5-26% of all clinical trials worldwide (Crystal, 2014; Lopez-Gordo et al., 2014).

1.3.1 Gene therapy and vaccine vectors

Adenoviruses, both human and non-human, have been extensively studied and employed as vaccines for decades, such as seen with HAdV-4 and HAdV-7 live oral vaccines given to military recruits (Top Jr et al., 1971). Numerous preclinical and clinical trials have occurred using recombinant AdV vectors against deadly human diseases including HIV (Seaman et al.,
2005; Kibuuka et al., 2010; Hammer et al., 2013), influenza (Gurwith et al., 2013), tuberculosis (Smaill et al., 2013), anthrax (Kasuya, 2005), and Ebola (Sullivan et al., 2003; Ledgerwood et al., 2014; Wong et al., 2015). Many of these AdV vectors have shown very promising results in vitro, in vivo, and in Phase I clinical trials.

In the field of cancer gene therapy, AdV vectors have been widely employed delivering a variety of therapeutic genes, such as the tumor suppressor genes p53, p16, antisense DNA, ribozymes, and single-chain antibodies, and the suicide genes of herpes simplex virus (HSV) (Douglas, 2007). Further, the first commercially approved gene therapy drug was based on a HAdV-5 vector expressing the p53 tumor suppressor gene (Peng, 2005). Named Gendicine, this product was approved by the State Food and Drug Administration of China for treatment of patients with head and neck squamous cell carcinoma (Wilson, 2005). Recombinant HAdV gene delivery is currently being applied towards a variety of cancers: Phase II treatment of advanced hepatocellular carcinoma with thymidine kinase (Beijing Chao Yang Hospital, 2015), Phase I treatment of ovarian cancer with thymidine kinase suicide gene and a somatostatin receptor (Kim et al., 2012), Phase I treatment of stage IV melanoma (National Human Genome Research Institute, 2015), and Phase II treatment of non-muscle invasive bladder cancer (Cold Genesys Inc, 2015).

While AdVs are the most characterized vector systems and with the highest clinical potential as vaccine and gene therapy vectors, these viruses are not without their own set of limitations. A major benefit, though also a limitation, of AdV vectors is their ability to elucidate strong innate and adaptive immune responses. It is estimated that over 80% of the adult human population has been naturally exposed to a variety of AdV serotypes commonly used as vectors (Garnett et al., 2002). Thus, clinical use of AdV vectors is often limited by a predisposed vector
immunity or rapid development of vector neutralizing antibodies resulting in the elimination of transduced cells, seen for example after repeat administration of an AdV vector expressing CFTR mRNA to cystic fibrosis patients (Harvey et al., 1999; Vogels et al., 2003; Nwanegbo et al., 2004, Bangari and Mittal, 2005). Furthermore, systematic inflammation due to the strong innate immune response can occur when high doses of virus are delivered intravenously, as evidenced by the tragic death of a patient administered a replication-incompetent HAdV-5 vector (Raper et al., 2003). Therefore, many efforts have been made to identify methods to avoid host antiviral immune responses in order to improve AdV vector safety without limiting efficacy, most of which have focused on modifying surface epitopes of the hexon, fiber, and knob (Lopez-Gordo et al., 2014).

Non-human AdV (NHAdV) vectors also represent viable alternatives for human therapy, as they not only elude pre-existing immunity, but also share many of the same desirable vector traits. Most NHAdV vectors exhibit low pathogenicity in their natural hosts, and when used outside of these hosts, the presence of pre-existing neutralizing antibodies, CD4+, and CD8+ T cells is avoided (Lopez-Gordo et al., 2014). NHAdVs that have shown the most promise as vectors are canine adenovirus 2 (CAdV-2) for neurological disorders (Klonjkowski et al., 1997; Bru et al., 2010), bovine adenovirus 3 (BAdV-3) in cancer gene therapy (Mittal et al., 1995; Tandon et al., 2012), porcine adenovirus 3 (PAdV-3) (Reddy et al., 1999; Sharma et al., 2011), chimpanzee adenovirus 68 (ChAdV-68) (Roy et al., 2004), FAdV-1 vectors for delivery of p53 and human IL-2 transgenes (Logunov et al., 2004; Tutykhina et al., 2008), FAdV-9 (Corredor and Nagy, 2010b; 2011), ovine adenovirus-7 (OAdV-7) (Löser et al., 2003; Tang et al., 2012), and murine adenovirus 1 (MAdV-1) (Cauthen et al., 1999; Lenaerts et al., 2012).

Furthermore, in veterinary medicine AdVs have also been used in the vaccination of
livestock and companion animals with great success. HAdV based vectors have been generated to express antigens such as the transmissible gastroenteritis virus (TGEV) spike protein (Torres et al., 1995), and the bovine coronavirus HE protein (Baca-Estrada et al., 1995). Recombinant HAdV-5 and CAdV-2 vectors encoding rabies virus glycoproteins resulted in efficient strategies for controlling rabies infection in animals (Hu et al., 2006; Rosatte et al., 2011). Recombinant FAdV-1 vectors expressing VP2 have been reported to protect poultry from IBDV (Francois et al., 2004), in addition to FAdV-8 vectors expressing neutralizing epitopes to IBDV (Greenall et al., 2010). PAdV-5 vectors encoding the spike protein of TGEV induce high levels of neutralizing antibodies in pigs (Tuboly and Nagy, 2001). BAdV-3 vectors encoding bovine herpesvirus (BHV) 1 glycoprotein D provide partial protection against challenge in calves (Reddy et al., 2000).

### 1.3.2 Recombinant FAdV-9 vectors

To date, most recombinant FAdVs have been generated by replacing non-essential regions of DNA at the right ends of FAdV-1, -8, -9, and -10 genomes (Sheppard et al., 1998a; Michou et al., 1999; Johnson et al., 2000; Francois et al., 2001; Ojkić and Nagy, 2001; 2003; Johnson et al., 2003). Further, these recombinant FAdVs (recFAdV) provided sufficient protection against viral challenge in the laboratory, hallmarked by recFAdV-1 expressing the VP2 protein of IBDV (Francois et al., 2004), recFAdV-8 expressing the S1 gene of infectious bronchitis virus (IBV) (Johnson et al., 2003), and recFAdV-8 expressing neutralizing fragments of the heavy and light chain variable domains of immunoglobulin to IBDV (Greenall et al., 2010). Investigations into the left terminus of the FAdV genome have uncovered additional non-essential regions in FAdV-1, however recFAdVs with deletions larger than 1.4 kb, or those that inactivated ORF1 (dUTPase), reduced the rate of virus replication (Michou et al., 1999; Francois
Research in our laboratory is focusing on the molecular characterization of a non-pathogenic strain of FAdV-9 (Accession #AF083975) and its development as a versatile vaccine vector platform. Initial studies focused on the sequence analysis of the genome, which is 45,063 bp, and performing transcriptional studies (Cao et al., 1998; Ojkić and Nagy, 2000; 2002). FAdV-9, like FAdV-1, lacks homologues to early regions 1, 3, and 4 (E1, E3, and E4) of mastadenoviruses, which are common sites for foreign gene accommodation in recombinant mammalian AdVs (Ojkić and Nagy, 2000; Davison et al., 2003). Further, FAdV-9 contains two TR sequences of unknown function in the right end of its genome, TR-1 (between nt 37,648-37,812) and TR-2 (between nt 38,807-40,561). In comparison to mastadenoviruses, the function of FAdV terminal ORFs at both the left and right ends of the genome are far less characterized. It is well understood that the central region of AdV genomes are well conserved, often referred to as genus-common genes, while the terminal ends are considered genus-specific and often encode genes required to modulate the host immune system (Davison et al., 2003). This fact, in combination with the unknown function of FAdV-9 TR-2, provided the basis to study the molecular function of FAdV-9 ORFs and the potential for FAdV-9 to be used as a vector.

An infectious FAdV-9 genomic clone was constructed by homologous recombination in Escherichia coli in order to engineer recFAdV-9 viruses, referred to as a FAdmid (Ojkić and Nagy, 2001). Briefly, genomic FAdV-9 DNA in the form of a plasmid is manipulated by homologous recombination in bacteria with an intermediate construct that contains a target cassette flanked by viral DNA. After recombination, the manipulated FAdmid can be isolated and linearized for transfection into chicken liver hepatoma (CH-SAH) cells, where the infectious DNA leads to the propagation of recFAdV. In initial studies, the coding sequence of enhanced
green fluorescence protein (EGFP) was introduced into the FAdV-9 genome using an intermediate construct, replacing TR-2. Replication competent virus was recovered after transfection of linearized FAdmid DNA, thus demonstrating that TR-2 was dispensable in vitro, as the recFAdV had similar growth characteristics to wt FAdV-9 (Ojkić and Nagy, 2001). Further analysis also demonstrated that TR-2 was dispensable for in vivo virus replication, as evident by distribution of virus in tissues and IgG antibody response measured by enzyme-linked immunosorbent assay (ELISA) in comparison to the wt FAdV-9 (Ojkić and Nagy, 2003).

Recent studies of FAdV-9 have focused on characterizing the left end of the genome. To elucidate the importance of ORFs, nine deletions (FAdV-9Δ1 - FAdV-9Δ9) were generated within nt 250-4200. Viable virus was recovered from four of the deletions (-9Δ1, -9Δ2, -9Δ4, and -9Δ7), and from analysis of these viruses it was determined that ORFs 24, 15, and 14 were important for in vitro replication. The packaging-signal motifs VI and VII, as well as the rightward oriented ORFs (0, 1, 1A, 1B, 1C, 2) were dispensable for in vitro virus replication shown by cytopathic effect (CPE) and virus growth kinetics (Corredor and Nagy, 2010a). Therefore, a 2,383 bp region at nt 400-2782 was identified as non-essential in the FAdV-9 genome. However, these deleted ORFs were important for virus replication in vivo, as evidenced by a decrease of viral genome copy numbers in tissues and significantly decreased virus shedding and IgG antibody response in chickens infected by one virus, FAdV-9Δ4 (Δ491-2782) (Figure 1.4).

The suitability of this region for foreign gene insertion was assessed by generating three recFAdVs encoding EGFP (Corredor and Nagy, 2010b). All recFAdVs (FAdV-9inEGFP, FAdV-9Δ1-EGFP, and FAdV-9Δ4-EGFP) exhibited similar growth kinetics and plaque morphology to the wt virus. EGFP was expressed in both avian and mammalian cells infected with recFAdVs
Figure 1.4. FAdV-9 genome and FAdV-9Δ4 deletion virus. The left end of the FAdV-9 genome (wild-type) consists of the first six open reading frames (ORFs) in the rightward orientation (ORFs 0, 1, 1A, 1B, 1C and 2) and five ORFs with leftward orientations (ORFs 24, 14, and, not shown 13, 12 and 15). FAdV-9 deletion viruses were generated by SgfI digestion and PCR-based site-directed mutagenesis reported in Corredor and Nagy (2010a). One of the replication competent deletion viruses, FAdV-9Δ4, containing a deletion between 491-2782 nt, was selected for *in vivo* studies.
based on spectrofluorometry and microscopy. CH-SA cells infected with FAdV-9inEGFP (a virus containing the EGFP coding cassette inserted into the non-essential region without deletion) expressed significantly higher levels of EGFP at 18 and 24 hours post-infection (h.p.i.), compared to FAdV-9Δ1-EGFP, and FAdV-9Δ4-EGFP, suggesting that some or all of the non-essential ORFs had a functional role on foreign gene expression (Corredor and Nagy, 2010b). Importantly, this study demonstrated the suitability of the left end non-essential region as an insertion site for transgenes, and the application of recFAdV-9 based vectors as recombinant vaccines for poultry or gene therapy vectors for mammalian systems.

The ability of FAdV-9inEGFP and FAdV-9Δ4-EGFP to stimulate a host immune response was tested in vivo (Corredor and Nagy, 2011). Chickens were inoculated intramuscularly with either recFAdV, and boosted at 2, 3, and 4 weeks post initial inoculation. All birds inoculated with recFAdVs had decreased viral shedding in the feces, and a decrease in IgG response to FAdV-9, as previously described (Corredor and Nagy, 2010a). Antibody response to EGFP was measured by ELISA and detected between 3-7 weeks post inoculation, with the highest levels of antibody being detected in birds inoculated with FAdV-9Δ4-EGFP. These results provided further evidence that the non-essential left end of FAdV-9 was suitable for foreign gene insertion, and foreign genes in this site could stimulate a host immune response making FAdV-9 vectors a viable poultry vaccine platform.

It is apparent that FAdV-9 based vectors have the potential to not only be excellent vaccine vectors for poultry, but also gene delivery vectors for a variety of mammalian systems. Corredor and Nagy (2011) demonstrated that intramuscular inoculation of poultry with recFAdVs stimulates a host immune response to a foreign transgene (EGFP), but it remained unclear whether this response was significant. Furthermore, Deng et al. (2013) demonstrated that
FAdV-9Δ4 could be given orally to chickens and stimulate the host immune response as previously described. A recFAdV-9Δ4 containing the coding sequence for highly pathogenic avian influenza (AI) H5N1 hemagglutinin (HA) under the cytomegalovirus (CMV) promoter was developed and tested in vitro (Yang et al., unpublished). As expected, FAdV-9Δ4-HA viruses were able to express high levels of biologically active HA. Interestingly, HA expression measured by Western immunoblot was found to be orientation dependent, in that viruses containing rightward oriented cassettes expressed higher levels of total protein compared to those in the leftward orientation (Yang et al., unpublished). Furthermore, data from an industry partner provided evidence that FAdV-9Δ4-HA inoculation stimulates a protective immune response against a highly pathogenic H5N1 influenza virus challenge (Gay et al., 2015). After a decade of development it is clear that FAdV-9 is a promising vaccine vector for poultry, and the ability to transduce mammalian cells warrants study on the use of FAdV as a vector which can circumvent AdV immunity in human gene therapy.

1.4 Elements regulating gene expression

1.4.1 Promoters

Promoters are gene elements that drive gene expression by acting as a binding site for transcription factors and RNA polymerase during the initiation of transcription. In the context of vaccine vectors, promoters regulate the specificity of expression, generally restricted to the 100–200 bp preceding the transcription start site. According to Gurunathan et al. (2000), the level of gene expression in vivo can be correlated with the immune response induced by the corresponding vaccines. It is therefore important to select an appropriate promoter for optimal expression when designing an expression system. Generally, four types of promoters are considered during a vector design: constitutively active eukaryotic cell and viral promoters,
inducible promoters, tissue-specific promoters, and host endogenous promoters.

High-level expression promoters are often used when there is no need for tightly regulated expression; such is the case with most vaccine approaches or proof-of-principle studies. The most widely used promoter for transgene expression is the immediate early CMV promoter, and this is due to its strong constitutive activity in a variety of cell types (Boshart et al., 1985; Qin et al., 2010; Gray et al., 2011). The strength of the CMV promoter can be attributed to its TATA box and enhancer elements located upstream of the transcription initiation site, as well as its cyclic-AMP response elements (Boshart, et al., 1985; Hunninghake et al., 1989). Other constitutively active promoters often used in vector systems the human elongation factor 1 alpha (EF1α), chicken β-actin, and its derivative the synthetic CMV enhancer/chicken β-actin (CAG), the β glucuronidase (GUSB), and ubiquitin C (UBC) (Fregien and Davidson, 1986; Kim et al., 1990; Niwa et al., 1991; Husain et al., 2009; Qin et al., 2010; Norrman et al., 2010).

A recent study by Qin et al. (2010) systematically compared the behaviors of a variety of common constitutive promoters across multiple cell lines under the same experimental conditions. Specifically, six promoters were studied, including the CMV, EF1α, and CAG promoters using lentivirus expression vectors containing a fluorescent reporter gene. The results showed that both the EF1α and CAG promoters consistently expressed the transgene in a variety of cell types, meanwhile CMV activity level varied greatly between cell types. Some of this variability in expression by the CMV promoter may be a result of promoter silencing by cellular methylation (Teschendorf et al., 2002, Brooks et al., 2004; Meilinger et al., 2009).

While Qin et al. (2010) are the first group to compare different promoters under strict experimental conditions, it is evident from their findings that the suitability of a promoter for a particular experiment and host should be considered.
The tetracycline-based transcription regulation system is the most commonly used inducible promoter, and has already been tested in lentivirus vectors, recombinant adenovirus-associated viruses (rAAVs) and HSV vectors (Goverdhana et al., 2005). This system is preferable for clinical gene therapy, as its regulation is well characterized. Though the inducer is a common antibiotic, its use would not be suitable for a vaccination protocol. Host specific promoters should be considered when designing a vector system, and in the context of FAdV-9 vector optimization, it may be advantageous to test both native chicken promoters, such as chicken β-actin, and avian viral promoters, such as the fowlpox virus (FPV) early/late promoter (L2R) (Zantinge et al., 1996), in addition to high-expression constitutive promoters, such as CMV, EF1α, and CAG.

1.4.2 Elements important for efficient expression

1.4.2.1 Kozak sequence

The Kozak sequence occurs in eukaryotic mRNA and has the consensus G/ANNAATGG. This sequence plays a major role in the initiation of translation, recruiting the ribosome to the ATG start codon of the mRNA (Kozak, 1987). Point mutations in the nucleotides surrounding the ATG start codon have been shown to modulate translation efficiency (Kozak, 1986). A purine residue (A or G) in -3 position has a dominant effect on expression, while changing the -3 position from a purine to a pyrimidine (C or T) reduces expression levels by up to 95%. Furthermore, when the -3 position is changed to a pyrimidine the sequence becomes more sensitive to changes in the -1, -2 and +4 positions.

1.4.2.2 Polyadenylation signals

On mRNAs, the poly(A) tail protects the mRNA molecule from enzymatic degradation in the cytoplasm and aids in transcription termination, export of the mRNA from the nucleus, and
translation (Guhaniyogi and Brewer, 2001). In eukaryotes, polyadenylation occurs at the consensus sequence AAUAAA in the mRNA. The poly(A) polymerase cleaves the mRNA, adding a string of A residues, anywhere from 50–250 residues long, to the 3'-end of an RNA transcript (Proudfoot, 1991; Wahle and Keller, 1992; Balbo and Bohm, 2007). In expression vectors, the polyadenylation sequence is found immediately following the reporter gene stop codon. Commonly used polyadenylation sequences are derived from the Simian virus 40 (SV40) early and late genes, or the bovine growth hormone (bGH) gene. Optimal expression usually involves the bGH or SV40 late poly(A), which have been shown to be 3-5 fold more efficient at generating high levels of steady-state mRNA than the SV40 early poly(A) (Carswell and Alwine, 1989; Proudfoot, 1991). In vectors that require two or more poly(A) sites, such as dual expression vectors containing two transgenes, non-homologous poly(A) signals should be used to reduce the chances of recombination within the vector, for example both bGH and SV40 late poly(A).

1.4.2.3 Enhancer regions

Enhancers are cis-acting DNA sequences that, although not necessary for expression, act to increase gene expression induced by a promoter through the recruitment of transcription factors and formation of chromatic loops (Krivega and Dean, 2012; Pennacchio et al., 2013). In vector design, they can be added to a construct in any region upstream of the promoter or even within an intron upstream of the transcriptional start site (Petitclerc, 1995; Pennacchio et al., 2013). The synergistic effects of promoters and enhancer elements are often cell type dependent (Wenger et al., 1994). The CMV enhancer, present upstream of the CMV promoter at nt -598 to -568, is commonly used to increase transgene expression in plasmids and viral vectors (Boshart et al., 1985). Studies showed that the CMV enhancer increases promoter driven transgene
expression, independent of the CMV promoter, in a variety of cell lines and therefore is a powerful tool for improving expression (Liu et al., 2004; Gruh et al., 2008).

1.4.2.3 Post-transcriptional regulatory elements

One method to overcome low level of transgene expression is the introduction of post-transcriptional regulatory elements that modulate the recombinant mRNA transport from the nucleus to the cytoplasm, mRNA stability, and translation efficiency (Hlavaty et al., 2005). Such elements include the mouse RNA transport element (RTE) (Nappi, 2001), the constitutive transport element of the simian retrovirus type 1 (Zolothukin et al., 1994; Gruter, 1998), and the 5’ untranslated region of the human heat shock protein 70 (Huez et al., 1998; Vivinus et al., 2001). Another element that enhances gene expression is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (Donello et al., 1998). WPRE is a noncoding cis-acting RNA sequence that increases the stability and extranuclear transport of mRNA to the cytoplasm, resulting in enhanced levels of mRNA for translation and protein production (Zufferey et al., 1999). The combination of WPRE with a strong promoter can increase transgene expression 2-50 fold (Zufferey et al., 1999; Klein et al., 2002a,b; Xu et al., 2003), and in the context of viral vaccine vector, including adenovirus based (Xu et al., 2003), has led to an increase in host immune response (Pertl et al., 2003; Garg et al., 2004).

1.4.3 Reporter genes

Reporter genes are those that encode an easily measurable trait, such as fluorescence, and are used to investigate the expression of other genes/promoter sequences for which functional assays are not available or for which measurement is difficult, generally cis-acting transcriptional elements (Rosenthal, 1987). Generally, a reporter gene is often manipulated through the construction of either a genetic fusion, where expression of the target gene also drives expression
of the reporter gene product, or through dual expression, where both reporter gene and target
gene are controlled by individual promoter/enhancer sequences. Measuring reporter gene
products provides a direct readout for expression levels of a target gene, therefore reporter genes
represent a valuable tool especially when studying and trying to optimize gene expression
(Slauch and Silhavy, 1991; Slauch et al., 1994; Schenborn and Groskreutz, 1999; Merrell and
Camilli, 2000).

Chloramphenicol acetyltransferase (CAT) is the most characterized reporter gene (Kain
and Granguly, 2001). A prokaryotic enzyme, CAT catalyzes the transfer of acetyl-coenzyme A
to chloramphenicol, and in most eukaryotic cells provides minimal competing activities,
resulting in low background readings (Gorman, 1982). In mammalian cells, CAT appears to be
very stable (Thompson et al., 1993), however most CAT assays require expensive radioactive
substrates, such as $^{14}$C-labelled chloramphenicol. In addition, CAT assays are both time
consuming and less sensitive compared to nonisotopic reporter systems such as fluorescence and
luminescence (Kain and Granguly, 2001). CAT reporter genes have been used in adenovirus
studies including HAdV-5 (Huang and Flint, 2003; Blackwood and Argyle, 2004), HAdV-7
(Abrahamsen et al., 1997), and FAdV-10 analyzing the major late promoter (MLP) (Sheppard et
al., 1998b).

The first nonisotopic reporter gene with widespread application in mammalian systems
was the luciferase gene from firefly Phontius pyralis (de Wet et al., 1987). This reporter gene
catalyzes a bioluminescent reaction involving luciferin (substrate), ATP, Mg2+, and molecular
O$_2$. The reaction results in a light signal that can be detected by a luminometer or a liquid
scintillation counter (Nguyen et al., 1988). The total emission of light is proportional to the
luciferase activity, and therefore provides an indirect estimate of gene translation. Luciferase is
sensitive to degradation by cellular proteases, giving it a half-life of approximately three hours in transfected mammalian cells (Thompson et al., 1993). Luciferase reporter genes have been used in adenovirus studies including HAdV-5 (Mittal et al., 1993), BAdV-3 (Mittal et al., 1995), and FAdV-1 (Michou et al., 1999; Goff et al., 2005).

Fluorescent proteins, such as green fluorescent protein (GFP), have become the most popular reporter genes in research. GFP was first discovered in the early 1960s when the bioluminescent properties of *Aequorea victoria* jellyfish were studied (Shimomura et al., 1962). Since then, GFP has been cloned and used to track expression both *in vitro* and *in vivo*, including that of FAdVs (Prasher et al., 1992; Chalfie et al., 1994; Corredor and Nagy 2010b). In addition, GFP has since been engineered to produce a vast number of new fluorescent proteins, such as EGFP and available in a variety of colours, each offering unique fluorescence characteristics (Matz et al., 1999; Shaner et al., 2004; 2008; Shcherbo et al., 2009). Fluorescence reporter genes have been utilized in AdV studies including HAdV-5 (Yang et al., 2013), FAdV-1 (Michou et al., 1999; Francois et al., 2001), and FAdV-9 (Ojkić and Nagy, 2001; Corredor and Nagy, 2010b).

One of the more versatile reporter genes is β-galactosidase, encoded from the lacZ gene of *E. coli*. Expression of this enzyme both *in vitro* and *in vivo* has been optimized with a variety of different commercial substrates, such as X-gal (Kain and Granguly, 2001). β-galactosidase as a reporter gene has been reported in HAdV-5 studies (Miller et al., 2004). Similarly, secreted alkaline phosphatase (SEAP) acts as a versatile reporter gene as unlike most reporter proteins, SEAP is secreted from transfected cells, and can be measured at any time by collecting transfected cell culture medium (Berger et al., 1988). The level of SEAP activity is measured by colorimetric assay using the substrate p-nitrophenyl phosphate, where it has been found that the
activity detected in cell culture medium is directly proportional to the changes of intracellular SEAP mRNA and protein (Cullen and Malim, 1992). In addition, SEAP is extremely heat stable, and pretreatment of transfected cells at 65°C will inactivate endogenous alkaline phosphatase, but not SEAP therefore eliminating background reactions. SEAP reporter genes have been used in adenovirus studies including HAdV-5 (Dlu et al., 2002), and FAdV-1 (Dlu et al., 2002; Goff et al., 2005).

Another reporter gene is the human growth hormone (hGH) gene which is exclusively secreted from somatotropic cells of the anterior pituitary gland, and therefore no endogenous activity is detected in other cell lines. HGH reporter assays have many of the same advantages as SEAP, however they are rarely used as they are costly, demonstrate relatively low sensitivity, and require radioimmunoassay to quantitate (Selden et al., 1986; Kain and Granguly, 2001). Lastly, the β-glucuronidase (GUSB) gene is often considered as a reporter gene in mammalian systems (Su et al., 2007), but specifically plant systems as plants lack endogenous GUSB activity (Kain and Granguly, 2001).

Depending on the nature of the experiment, several considerations should be evaluated when selecting a reporter gene. A reporter gene should be absent from the host in which it is being tested, if not easily distinguished from the endogenous form. Reporter genes should be detected by simple, rapid, sensitive, and cost-effective assays, in addition to having a broad linear range to facilitate measurement of both small and large changes in promoter activity. Expression of reporter genes must not alter the physiology of the recipient cells or organism, and ideally codon usage should be optimized for that host (Kain and Granguly, 2001). Moreover, the sensitivity of the assay should be considered when selecting a reporter gene, in addition to the equipment required to measure expression.
1.5 Research objectives and experimental design

To date, research in our laboratory has developed a system for the construction of recombinant viruses based on a non-pathogenic strain of FAdV-9. We demonstrated that both the right end TR-2 region (Ojkić et al., 2001) and a 2.4 kb region in the left end of the genome (Corredor et al., 2010a,b) are both non-essential for virus replication and suitable for the insertion of a foreign gene. Recombinant FAdVs expressing EGFP (Ojkić and Nagy, 2003; Corredor and Nagy, 2010b; 2011) and HA from highly pathogenic H5N1 viruses (Yang et al., unpublished) have been generated and evaluated both in vitro and in vivo. RecFAdVs not only express biologically active forms of these transgenes, but also induce a host immune response when administered intramuscularly or orally, thus demonstrating their capacity to be used as vaccine vectors for poultry. However, while recFAdVs do stimulate a host immune response, optimizing the expression of viruse-encoded transgenes as a way to maximize transgene-specific immune responses remains a priority.

The research presented in this thesis aims to increase FAdV-mediated expression of a foreign gene. It was hypothesized that:

1) Foreign gene expression by recFAdVs in vitro could be improved by optimizing the transgene expression cassette with high-expression promoters and regulatory elements

2) Optimized foreign gene expression by recFAdVs will stimulate a greater host immune response to the foreign gene

The first research objective was to compare and contrast the expression of EGFP in vitro in transfected avian cells. Under various avian and mammalian promoters, and in the presence or absence of an enhancer element, the expression of EGFP would be measured and compared to the promoter currently used to regulate transgene expression in recFAdVs, the CMV promoter. A plasmid dual-expression system was generated using firefly luciferase and deployed to normalize
EGFP expression from each promoter, thus accounting for experimental variables such as transfection efficiency and plasmid copy number. Normalized expression from each plasmid could be compared to that of a plasmid containing the CMV promoter, and provide insight into specific promoter activity in avian cell lines.

The second objective was to generate multiple recFAdV-9Δ4 viruses expressing EGFP under the promoters that performed best in the first objective. Once again, each virus would be compared to FAdV-9Δ4-CMV-EGFP by measuring protein expression by fluorometry, SDS-PAGE and Western immunoblots. The results presented in this thesis provide support towards my first hypothesis by addressing objectives one and two.

Although not presented in this thesis, the third objective of my research was to conduct an experiment in chickens to evaluate the immune response induced by each of these different promoter expression cassettes. Antibodies in sera, intestinal tissues, and lungs to both the FAdV-9Δ4 backbone and EGFP transgene would be measured by ELISA. Further, shedding of recFAdV in the feces of inoculated birds would be evaluated.
Chapter 2. Material and Methods

2.1 Cell culture and viruses

CH-SAH cells were maintained in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham (DMEM-F12) (Sigma) supplemented with 200 mM L-glutamine and 100 U/ml penicillin-streptomycin (PenStrep, Sigma) and 10% non-heat inactivated fetal bovine serum (FBS, Sigma) as previously described (Alexander et al., 1998). Recombinant FAdVs were generated based on the FAdV-9Δ4 deletion virus described by Corredor and Nagy (2010a). Propagation of all viruses was carried out in CH-SAH cells (Solvay, Mendota Heights, MN) as described by Alexander et al. (1998).

2.2 General DNA manipulation

2.2.1 Bacterial cultures and plasmid isolation

E. coli DH5α cells were the bacterial host for all plasmids described, while E. coli BJ5183 cells were used for homologous recombination to generate recombinant FAdmids. Bacterial cultures were grown on selective Luria-Bertani (LB, Difco) liquid or agar (16 mg/ml) growth medium containing ampicillin (100 µg/ml, Sigma) at 37ºC. Single E. coli colonies were picked, inoculated in 5 ml of LB medium supplemented with ampicillin to select for growth of bacteria containing a transformed ampicillin resistant plasmid. Incubation and growth occurred for approximately 16 hours. Chemically (CaCl$_2$) competent DH5α E. coli were prepared and transformed with either 10 µl of ligation product or 1 µl purified plasmid DNA (100 ng/µl) (Sambrook and Russel, 2001). All plasmids were isolated using either the EZ-10 Spin Column Plasmid DNA Mini-prep kit (Bio Basic) or the PureLink HiPure Plasmid Midiprep kit (Invitrogen) (for plasmids larger than 40 kb in size or when a high concentration of DNA was needed) as per the manufacturer’s protocol.
2.2.2 PCR and restriction enzyme digestion

DNA was amplified by polymerase chain reaction (PCR) during the cloning of all dual-expression constructs and recombinant viruses. For all cloning, PCR amplification was conducted using a Kod Hot Start Polymerase kit (Novagen). However, Taq polymerase (Sigma) was used when screening a plasmid by PCR. Unless stated otherwise, the PCR conditions for both Kod and Taq polymerases are summarized in Table 2.1. All PCR reactions were carried out with a Mastercycler Pro (Eppendorf).

Restriction enzyme (RE) digestions were carried out for both Fast Digest enzymes (Fermentas) and enzymes from New England BioLabs (NEB) as per the manufacturers’ protocol (per enzyme). Digestion reactions were always performed at 37ºC, and samples were heat inactivated in a Mastercycler Pro (Eppendorf) thermocycler.

Unless otherwise stated, all DNA samples were subjected to electrophoresis at 100V in 0.8% agarose gels containing 1x RedSafe™ (iNtRON Biotechnology). 6X DNA loading buffer [0.25% (w/v) bromophenol blue, 40% sucrose (w/v) in water] and 1X Tris-acetate-EDTA (TAE) was the buffer for electrophoresis of DNA samples.

2.2.3 Plasmid construction and transformation

After RE digestion and gel purification, PCR amplified DNA and plasmid were ligated with T4 DNA ligase (Invitrogen). Unless stated otherwise, ligations were performed at a molar ratio of 1:1 insert to vector, overnight at 16ºC. Fifty µl of CaCl₂ competent DH5α E. coli were mixed with 10 µl of ligation product or 1 µl purified plasmid DNA (100 ng/µl). Competent cells and DNA were incubated on ice for 30 min, then heat shocked at 42ºC for 1 min. Cells were recovered on ice for 3 min and 500 µl of super optimal broth with catabolite repression (SOC, Bio Basic) medium was added to each microcentrifuge tube. The cells were incubated with
Table 2.1. PCR conditions using either Kod or Taq polymerase as per the manufacturer’s protocol.

**Kod Polymerase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Target size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;500 bp</td>
</tr>
<tr>
<td>Activation</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>70°C for 10s/kb</td>
</tr>
<tr>
<td>Repeat Steps 2-4</td>
<td></td>
</tr>
</tbody>
</table>

**Taq Polymerase**

<table>
<thead>
<tr>
<th>Step</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>95°C for 5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C for 1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>Lowest Primer Tm°C for 30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>70°C for 1 min/kb</td>
</tr>
<tr>
<td>Repeat Steps 2-4</td>
<td></td>
</tr>
</tbody>
</table>

25 cycles
agitation at 37°C for 1 hr, then centrifuged and resuspended in 100 µl of LB broth. The entire volume was spread on an LB agar plate containing ampicillin.

Homologous recombination was in *E. coli* BJ5183 cells. Two µg of both promoter cassettes and linear FAdV-9Δ4 DNA were mixed in 100 µl of chemically competent BJ5183 cells. Mixtures were left on ice for 15 min, followed by a heat shock at 42°C for 1 min. Cells were recovered on ice for 20 min, and 1 ml of SOC medium was added to each microcentrifuge tube and transferred into a 5 ml glass culture tube. Cells were incubated for 2 hrs at 37°C with agitation, then centrifuged and resuspended in 100 µl of LB broth. The entire volume of cells was spread onto a LB agar plate containing ampicillin.

2.2.4 Sequencing

All PCR products and plasmids were purified and sequenced by the ABI 3730 DNA sequencer (Laboratory Services Division, Guelph, ON). Sequence data were analysed using SnapGene Viewer (GSL Biotech).

2.3 Analysis of promoter expression

2.3.1 Generation of dual-expression plasmid constructs

The activity of five promoters (CMV, CAG, EF1α, β-actin, and L2R) and one enhancer element (WPRE) was compared by measuring the expression of EGFP relative to firefly luciferase under the SV40 promoter in transfected CH-SAH cells. The plasmids, pCI-Neo (Promega), pCAG-Puro, and pEF1α-Puro, were provided by Dr. Sarah Wootton (University of Guelph). Dual-expression plasmids were generated using the plasmid pCI-Neo as a backbone (Figure 2.1), which contained the CMV promoter along with numerous unique RE sites. Both the CAG and EF1α promoters were sub-cloned from pCAG-Puro and pEF1α-Puro, respectively, into pCI-Neo using SpeI and EcoRI. The presence of each promoter was confirmed by sequencing
Figure 2.1. Schematic representation of pCI-Neo. The plasmid pCI-Neo (Promega) was chosen to create dual expression constructs due to its unique restriction enzyme sites in and before the multiple cloning site, as well as the neomycin resistance gene.
with the primer pCI-Neo-F (Table 2.2). EGFP was amplified by PCR from pEGFP-N1 (Clontech Laboratories, Inc) with primers EGFP-F and EGFP-R (Table 2.2) at an annealing temperature of 60ºC. The resulting PCR product was gel extracted using the Wizard Plus SV Miniprep DNA Purification Kit (Promega). Both EGFP PCR product and pCI-Neo based plasmids (containing the CMV, CAG, or EF1α promoter) were subjected to double digestion with EcoRI and NotI for 1 hr at 37ºC. Both digested plasmid and PCR product were then separated in an agarose gel, extracted using Wizard Plus SV Miniprep DNA Purification Kit, and ligated overnight at 4ºC. Following transformation into E. coli DH5α cells and growth on LB-amp plates, colonies were PCR screened for the presence of the EGFP fragment with primers EGFP-F and EGFP-R. All positive colonies were confirmed by sequencing with EGFP-I-F primer (Table 2.2), resulting in the plasmids pCMV-EGFP, pCAG-EGFP, and pEF1α-EGFP.

The β-actin promoter was PCR amplified from pCAG-Puro using the primers βactin-F and βactin-R (Table 2.2) with an annealing temperature of 60ºC. The resulting PCR product was gel extracted with the Wizard Plus SV Miniprep DNA Purification Kit. Both β-actin PCR product and pCAG-EGFP were subjected to double digestion with EcoRI and NotI for 1 hr at 37ºC. Digested plasmid and PCR product were then separated by electrophoresis and extracted using Wizard Plus SV Miniprep DNA Purification Kit, and ligated overnight at 4ºC. Following transformation into E. coli DH5α cells and growth on LB-amp plates, colonies were screened with RE for the presence of β-actin. All positive colonies were confirmed by sequencing using both pCI-Neo-F and EGFP-I-F primers (Table 2.2), resulting in the plasmid pβactin-EGFP.

The FPV L2R promoter was PCR amplified from pE68 (Zantinge et al., 1996) with the primers L2R-F and L2R-R (Table 2.2) at an annealing temperature of 60ºC. The resulting PCR product was gel extracted using the Wizard Plus SV Miniprep DNA Purification Kit. Both L2R
Table 2.2. Primers designed for the generation of dual expression constructs and recFAdVs. Restriction enzyme sites incorporated into each primer are underlined.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Tm (°C)</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCI-Neo-F</td>
<td>GGCTCATGTCCCAATATGACCGCCAT</td>
<td>61</td>
<td>-</td>
</tr>
<tr>
<td>EGFP-F</td>
<td>AAAAAAGAATTCACCATGGTGAGCAAGGGCGAG</td>
<td>58</td>
<td>EcoRI</td>
</tr>
<tr>
<td>EGFP-R</td>
<td>AAAAAAGCGGCCGCTTTACTTGTAACGCTCGTCCATGCC</td>
<td>59</td>
<td>NotI</td>
</tr>
<tr>
<td>EGFP-I-F</td>
<td>GAGAAGCGCGATCACATGTT</td>
<td>58</td>
<td>-</td>
</tr>
<tr>
<td>βactin-F</td>
<td>AAAAAAACTAGTTCAGGTGAGGTAGCCACGT</td>
<td>65</td>
<td>SpeI</td>
</tr>
<tr>
<td>βactin-R</td>
<td>AAAAAGAATTCGCCGCCGCCGCTTCGCTT</td>
<td>71</td>
<td>EcoRI</td>
</tr>
<tr>
<td>L2R-F</td>
<td>AAAAAAACTAGTTCAGGTGAGGTGAGCCACGT</td>
<td>55</td>
<td>SpeI</td>
</tr>
<tr>
<td>L2R-R</td>
<td>AAAAAGAATTCAGGTGAGGTGAGCCACGT</td>
<td>55</td>
<td>EcoRI</td>
</tr>
<tr>
<td>Luc-F</td>
<td>AAAAAACCTAGGTTGGCAATCCGGGACTGTTGGT</td>
<td>59</td>
<td>AvrII</td>
</tr>
<tr>
<td>Luc-R</td>
<td>AAAAAATTCGAGCGCCGCCCTCGGGTACTCTTAG</td>
<td>58</td>
<td>BstBI</td>
</tr>
<tr>
<td>SV40-F</td>
<td>GGCCAATCTAGCTGAGAAGTATGGAGGCCCTAAGCTGGAA</td>
<td>56</td>
<td>NotI</td>
</tr>
<tr>
<td>WPRE-F</td>
<td>AAAAAGCGGCCGCTCAACTCTGTGAGATTACAAATTTTGAG</td>
<td>56</td>
<td>NotI</td>
</tr>
<tr>
<td>WPRE-R</td>
<td>AAAAAGCGGCCGCGCCCAAAGGGAGATCCGCGC</td>
<td>58</td>
<td>NotI</td>
</tr>
<tr>
<td>VF1-F</td>
<td>AAAAAAGATCCTGACAGGTAGGCTGACGTG</td>
<td>53</td>
<td>BglII</td>
</tr>
<tr>
<td>VF1-R</td>
<td>AAAAAACACTAGTAAACCGCAATACCCCGAATACCCAG</td>
<td>53</td>
<td>SpeI</td>
</tr>
<tr>
<td>VF2-F</td>
<td>AAAAAACGTGAAATAAAAGACTCAGAGAAGCCTG</td>
<td>54</td>
<td>MfeI</td>
</tr>
<tr>
<td>VF2-R</td>
<td>AAAAAAGGTACCCCGCCAAGAGATTTAAAA</td>
<td>53</td>
<td>KpnI</td>
</tr>
<tr>
<td>E1-F</td>
<td>ACGATGCGGCTGAGATGCGTGGG</td>
<td>53</td>
<td>-</td>
</tr>
<tr>
<td>E1-R</td>
<td>CCCCCGCCGAGAATTTAAAA</td>
<td>52</td>
<td>-</td>
</tr>
</tbody>
</table>
PCR product and pCMV-EGFP were subjected to double digestion with EcoRI and NotI for 1 hr at 37°C. Both digested plasmid and PCR product were then separated by electrophoresis, extracted using Wizard Plus SV Miniprep DNA Purification Kit, and ligated overnight at 4°C. Following transformation into *E. coli* DH5α cells and growth on LB-amp plates, colonies were screened with RE for the presence of L2R. All positive colonies were confirmed by sequencing with both pCI-Neo-F and EGFP-I-F primers (Table 2.2), resulting in the recovery of the plasmid pL2R-EGFP.

Firefly luciferase was PCR amplified from pGL4.17 (Promega) with primers Luc-F and Luc-R (Table 2.2) at an annealing temperature of 55°C. The resulting PCR product (1.6 kb) was gel extracted using the Wizard Plus SV Miniprep DNA Purification Kit. Both luciferase PCR product and promoter plasmids were subjected to double digestion with AvrII and BstBI for 1 hr at 37°C. Digested plasmid and PCR product were then separated by electrophoresis, removing the neomycin resistance (NeoR) cassette from each plasmid, extracted using Wizard Plus SV Miniprep DNA Purification Kit, and ligated overnight at 4°C. Following transformation into *E. coli* DH5α cells and growth on LB-amp plates, colonies were PCR screened for the presence of luciferase. All positive colonies were confirmed by sequencing using SV40-F primer (Table 2.2).

Five additional dual-expression plasmids were generated to include WPRE. The WPRE element was PCR amplified from pWPRE (Dr. Sarah Wootton, University of Guelph) using the primers WPRE-F and WPRE-R (Table 2.2) with an annealing temperature of 55°C. The PCR product was gel extracted using the Wizard Plus SV Miniprep DNA Purification Kit. Both WPRE PCR product and promoter were subjected to digestion with NotI for 1 hr at 37°C. After digestion, plasmids were treated with alkaline phosphatase (calf intestinal, NEB) as per the manufacturer’s protocol to prevent re-ligation. Both digested/dephosphorylated plasmid and PCR
product were then separated by electrophoresis, DNA was extracted using Wizard Plus SV Miniprep DNA Purification Kit, and ligated overnight at 4°C. Following transformation into *E. coli* DH5α cells and growth on LB-amp plates, colonies were PCR screened for the presence of WPRE. All positive colonies were confirmed by sequencing using EGFP-I-F (Table 2.2). All plasmids generated in this study and their purposes are listed in Table 2.3.

### 2.3.2 Transfection of chicken hepatoma cells

The expression of EGFP was measured by transfecting CH-SAH cells with the dual-expression plasmids. Cells were seeded in 35 mm dishes at a density of 1.8x10^6 cells/dish and incubated at 37°C with 5% CO₂. Lipofectamine 2000 (Invitrogen) was used to transfect all constructs according to the manufacturer’s recommendation. Briefly, 2 µg of dual-expression plasmid and 5 µl of Lipofectamine were incubated in separate 50 µl aliquots of Opti-Mem medium (Gibco) for 5 minutes, then mixed and incubated together for 20 minutes. During this period of time, the medium was removed from each dish, and the cell monolayers were washed two times with phosphate buffered saline (PBS). Two ml of DMEM-F12 (5% FBS) without antibiotics was added to each dish. After 20 minutes, the plasmid-lipofectamine mixtures were added to the 35 mm dishes and incubated for 6 hrs at 37°C in the presence of 5% CO₂. This was repeated for all ten dual-expression plasmids. After 6 hrs, the medium was removed and fresh DMEM-F12 (5% FBS) was added. Every 12 hours post-transfection (h.p.t.), EGFP expression was confirmed by fluorescence microscopy and whole cell lysates were collected. Monolayers were washed with PBS, trypsin was added, and the cells were resuspended in DMEM-F12 (10% FBS). The cells were then centrifuged in 15 ml conical tubes (Nunc®), the supernatant was
Table 2.3. Vectors used and generated in this study and their key features.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Size (bp)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCI-Neo</td>
<td>5,472</td>
<td>Dual expression plasmid backbone with CMV promoter</td>
</tr>
<tr>
<td>pCAG-Puro</td>
<td>6,018</td>
<td>CAG promoter for cloning</td>
</tr>
<tr>
<td>pEF1α-Puro</td>
<td>5,840</td>
<td>EF1α promoter for cloning</td>
</tr>
<tr>
<td>pE68</td>
<td>6,500</td>
<td>L2R promoter for cloning</td>
</tr>
<tr>
<td>pEGFP-N1</td>
<td>4,733</td>
<td>EGFP cassette for cloning</td>
</tr>
<tr>
<td>pGL4.17</td>
<td>5,599</td>
<td>Firefly luciferase cassette for cloning</td>
</tr>
<tr>
<td>pCMV-EGFP-Luc</td>
<td>7,003</td>
<td>Dual expression cassette for CMV-EGFP</td>
</tr>
<tr>
<td>pCAG-EGFP-Luc</td>
<td>7,760</td>
<td>Dual expression cassette for CAG-EGFP</td>
</tr>
<tr>
<td>pEF1α-EGFP-Luc</td>
<td>7,566</td>
<td>Dual expression cassette for EF1α-EGFP</td>
</tr>
<tr>
<td>pβactin-EGFP-Luc</td>
<td>6,344</td>
<td>Dual expression cassette for βactin-EGFP</td>
</tr>
<tr>
<td>pL2R-EGFP-Luc</td>
<td>6,179</td>
<td>Dual expression cassette for L2R-EGFP</td>
</tr>
<tr>
<td>pWPRE</td>
<td>3,511</td>
<td>WPRE cassette for cloning</td>
</tr>
<tr>
<td>pCMV-EGFP-WPRE-Luc</td>
<td>7,546</td>
<td>Dual expression cassette for CMV-EGFP-WPRE</td>
</tr>
<tr>
<td>pCAG-EGFP-WPRE-Luc</td>
<td>8,295</td>
<td>Dual expression cassette for CAG-EGFP-WPRE</td>
</tr>
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<td>pEF1α-EGFP-WPRE-Luc</td>
<td>8,101</td>
<td>Dual expression cassette for EF1α-EGFP-WPRE</td>
</tr>
<tr>
<td>pβactin-EGFP-WPRE-Luc</td>
<td>6,879</td>
<td>Dual expression cassette for βactin-EGFP-WPRE</td>
</tr>
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<td>pL2R-EGFP-WPRE-Luc</td>
<td>6,714</td>
<td>Dual expression cassette for L2R-EGFP-WPRE</td>
</tr>
<tr>
<td>pHMR-CMV-EGFP</td>
<td>8,203</td>
<td>Intermediate construct for homologous recombination with pFAdV-9Δ4</td>
</tr>
<tr>
<td>pHMR-CMV-EGFP-WPRE</td>
<td>8,736</td>
<td>Intermediate construct for homologous recombination with pFAdV-9Δ4</td>
</tr>
<tr>
<td>pHMR-CAG-EGFP</td>
<td>8,960</td>
<td>Intermediate construct for homologous recombination with pFAdV-9Δ4</td>
</tr>
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<td>pHMR-CAG-EGFP-WPRE</td>
<td>9,484</td>
<td>Intermediate construct for homologous recombination with pFAdV-9Δ4</td>
</tr>
<tr>
<td>pHMR-EF1α-EGFP</td>
<td>8,766</td>
<td>Intermediate construct for homologous recombination with pFAdV-9Δ4</td>
</tr>
<tr>
<td>pHMR-EF1α-EGFP-WPRE</td>
<td>9,290</td>
<td>Intermediate construct for homologous recombination with pFAdV-9Δ4</td>
</tr>
<tr>
<td>pFAdV-9Δ4</td>
<td>44,971</td>
<td>FAdmid backbone Δ491-2,782</td>
</tr>
<tr>
<td>pFAdV-9Δ4-CMV-EGFP</td>
<td>46,826</td>
<td>FAdmid containing CMV-EGFP in Δ4 site</td>
</tr>
<tr>
<td>pFAdV-9Δ4-CMV-EGFP-WPRE</td>
<td>47,350</td>
<td>FAdmid containing CMV-EGFP-WPRE in Δ4 site</td>
</tr>
<tr>
<td>pFAdV-9Δ4-CAG-EGFP</td>
<td>47,583</td>
<td>FAdmid containing CAG-EGFP in Δ4 site</td>
</tr>
<tr>
<td>pFAdV-9Δ4-CAG-EGFP-WPRE</td>
<td>48,107</td>
<td>FAdmid containing CAG-EGFP-WPRE in Δ4 site</td>
</tr>
<tr>
<td>pFAdV-9Δ4-EF1α-EGFP</td>
<td>47,389</td>
<td>FAdmid containing EF1α-EGFP in Δ4 site</td>
</tr>
<tr>
<td>pFAdV-9Δ4-EF1α-EGFP-WPRE</td>
<td>47,913</td>
<td>FAdmid containing EF1α-EGFP-WPRE in Δ4 site</td>
</tr>
</tbody>
</table>
removed, and the cell pellet was resuspended in 500 µl PBS and frozen at -80°C. Transfected cell samples frozen at -80°C were freeze-thawed three times. The cell debris was spun down at 12,000 rpm in a microcentrifuge for 10 minutes at 4°C. Supernatant was transferred to a fresh microcentrifuge tube and the protein concentration was determined at 280 nm using a Nanodrop 2000 (Thermo Scientific). All samples were adjusted to a protein concentration of 1 µg/µl.

2.3.3 Fluorescence microscopy

Both transfected and infected CH-SAH cells were monitored by fluorescence microscopy with a Zeiss fluorescence microscope (Carl Zeiss) with FITC optics.

2.3.4 Measuring reporter gene expression

The expression of EGFP was quantified by spectrofluorometry using a GloMax®-Multi (Promega) microplate reader. Briefly, 50 µg of protein lysate was added in triplicate to a flat-bottomed black 96-well plate (Corning). Fluorescence of EGFP was measured in a GloMax®-Multi (Promega) microplate reader at 480 nm excitation and 528 nm emission wavelengths. The three readings were averaged to give one fluorescence value per sample.

Luciferase expression was determined using a Pierce Firefly Luciferase Glow Assay kit (Thermo Fisher Scientific) as per the manufacturer’s protocol. Briefly, 25 µg of protein lysate was added in triplicate to a flat-bottomed black 96-well plate. Fifty µl of luciferase assay substrate was manually added to each replicate, mixed well, and incubated for 15 minutes protected from light before measuring luminescence in a GloMax®-Multi microplate reader. The readings were averaged to give one luminescence value per sample.

2.3.5 Normalizing promoter expression

Normalization of dual-expression constructs was performed to remove sample-to-sample variability of transfection efficiency (Schagat et al., 2007). The fold change in activity (Δfold)
was determined between promoter constructs and pCMV-EGFP-Luc. To begin, for each specific time-point and repetition the average fluorescence and luminescence values were determined for each plasmid construct, from which a ratio of fluorescence over luminescence (F/R) was calculated. Activity level was compared to the CMV promoter, currently used in recFAdVs, therefore the F/R ratio of CMV was set to a value of 1.0 (was divided by its own F/R ratio). Each remaining construct F/R value was also divided by the F/R value of CMV, resulting in a value representing the normalized Δfold. Average fold change between repetitions was compared between all constructs at each time-point.

2.4 Generation and characterization of recombinant viruses

2.4.1 Construction of intermediate constructs

Further analysis of EGFP expression in vitro was performed with recFAdVs containing the CMV, CAG, and Ef1α based expression cassettes. In previous studies, recFAdVs were recovered by homologous recombination between pFAdV-9Δ4 and the PCR amplified expression cassette containing viral flanking regions, isolated from an intermediate construct. The plasmid pleftΔ491-2,782 (pLΔ2.4) contains the left end Δ4 deletion site (Δ491-2,782 nt) of FAdV-9 with a SvaI RE site for blunt-cloning of a transgene (Corredor and Nagy, 2010b). In this study, a new intermediate construct system was developed by cloning viral flanking regions directly into the dual-expression plasmids, thus creating intermediate construct plasmids ready for homologous recombination with pFAdV-9Δ4 (pHMR). Viral genomic regions flanking the Δ4 deletion site of FAdV-9 were PCR amplified from the intermediate construct pLΔ2.4. The region left of the deletion site (VF1) was PCR amplified using 100 ng of pLΔ2.4 and primers VF1-F and VF1-R (Table 2.2) with an annealing temperature of 52°C. The resulting PCR product was gel extracted using the EZ-10 Spin Column Plasmid DNA Mini-prep kit. Both VF1
PCR product and all dual expression vectors were digested with SpeI for 1 hr, followed by digestion with BglII. Both digested plasmid and PCR product were then separated by electrophoresis, extracted using QIAEX II Gel Extraction kit (Qiagen), and ligated overnight at 16°C. Following transformation into *E. coli* DH5α cells and growth on LB-amp plates, colonies were PCR screened for the presence of the VF1 fragment with primers VF1-F and VF1-R. All positive colonies were confirmed by sequencing. Next, the region to the right of the deletion site (VF2) was PCR amplified using 100 ng of pLΔ2.4 and primers VF2-F and VF2-R (Table 2.2) with an annealing temperature of 52°C. The resulting PCR product was gel extracted with the EZ-10 Spin Column Plasmid DNA Mini-prep kit. Both VF2 PCR product and all dual expression vectors positive for the VF1 fragment were digested with KpnI for 1 hr, followed by digestion with MfeI. Both digested plasmid and PCR product were then separated by electrophoresis and extracted using QIAEX II Gel Extraction kit, and ligated overnight at 16°C. Following transformation into *E. coli* DH5α cells and growth on LB-amp plates, colonies were PCR screened for the presence of the VF2 fragment using VF1-F and VF2-R primers (Table 2.2) at an annealing temperature of 52°C, with an expected size of 2 kb plus the size of each EGFP expression cassette. A list of all six pHMR intermediate plasmids is presented in Table 2.3.

### 2.4.2 Generation of recombinant fowl adenoviruses

Recombinant FAdVs were generated to include the CMV, CMV-WPRE, CAG, CAG-WPRE, EF1α, and EF1α-WPRE expression cassettes using a method modified from Corredor and Nagy (2010b) (Figure 2.2). Expression cassettes flanked by viral DNA in the pHMR plasmids were recombined with pFAdV-9Δ4 to create new recombinant FAdmids, containing a promoter of interest and EGFP. To obtain promoter EGFP cassettes flanked by viral DNA sequences, intermediate pHMR constructs were subjected to PCR or RE digestion with
Figure 2.2. Generation of recombinant FAdV-9Δ4 viruses. (A) The EGFP expression cassette was amplified by PCR from an intermediate pHMR construct, or gel extracted after double digestion with BamHI and BglII. (B) pFAdV-9Δ4 was linearized and digested with Swal. (C) Both the EGFP cassette and the linearized pFAdV-9Δ4 was co-transformed into E. coli BJ5183 cells to undergo homologous recombination. (D) The resulting plasmid was transformed into E. coli DH5α cells, propagated, screened by NotI digestion, and eventually linearized with PacI to release the viral genome from the plasmid. The linear recombinant viral DNA was transfected into CH-SAH cells. (E) Recombinant virus was collected in the supernatant. This procedure was carried out for each EGFP expression cassette, resulting in the viruses: FAdV-9Δ4-CMV-EGFP, FAdV-9Δ4-CMV-EGFP-WPRE, FAdV-9Δ4-CAG-EGFP, FAdV-9Δ4-CAG-EGFP-WPRE, FAdV-9Δ4-EF1α-EGFP, and FAdV-9Δ4-EF1α-EGFP-WPRE.
BglII/BamHI. Cassettes containing the CMV promoter (pHMR-CMV-EGFP and pHMR-CMV-EGFP-WPRE) and EF1α promoter (pHMR- EF1α-EGFP and pHMR- EF1α-EGFP-WPRE) were PCR amplified. PCR was carried out with 200 ng of plasmid and primers E1-F and E1-R (Table 2.2) with an annealing temperature of 52°C, and the resulting PCR product was gel extracted using QIAEX II Gel Extraction kit. Cassettes containing the CAG promoter (pHMR-CAG-EGFP and pHMR-CAG-EGFP-WPRE) were double-digested using BglII/BamHI. Briefly, 5 µg of plasmid was digested with both enzymes for 1 hr at 37°C. Samples were separated by gel electrophoresis, and bands corresponding to CAG cassette (4.5 kb) or CAG-WPRE cassette (5 kb) were gel extracted using EZ-10 Spin Column DNA Gel Extraction kit. Next, 5 µg of pFAdV-9Δ4 was linearized with SwaI and ethanol precipitated. The concentration of all DNA obtained by PCR and RE digestion product was measured with a Nanodrop 2000 (Thermo Scientific). For each construct, 2 µg of promoter EGFP cassette and 2 µg of SwaI digested pFAdV-9Δ4 were co-transformed into E. coli BJ5183 cells. All resulting constructs were screened for recombination by NotI digestion followed by transformation into E. coli DH5α. These constructs were grown in LB medium, isolated using PureLink HiPure Plasmid Midiprep kit, digested with PacI, extracted and ethanol precipitated. CH-SAH cells were seeded into 25 mm² flasks at a density of 4.3x10⁶ cells/flask. Five µg of PacI digested DNA was transfected into the CH-SAH cells using 25 µl of Lipofectamine 2000. After 6 hrs, the transfection mixture was removed and fresh DMEM-F12 (5% FBS) was added, the cells were incubated at 37°C with 5% CO₂. Cells were monitored for the appearance of CPE for the next 5-7 days. When CPE appeared the cells were frozen and thawed three times, centrifuged, and the clarified supernatant containing recombinant FAdV-9Δ4 was stored as P0 stock at -80°C. Virus was subsequently passaged three times to obtain a high titre P4 stock. A list of recFAdVs is given in Table 2.4.
### Table 2.4. Viruses used and generated in this study and their key features.

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Size (bp)</th>
<th>Relation to FAdV-9 (%)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAdV-9Δ4*</td>
<td>42,772</td>
<td>94.90</td>
<td>FAdV-9 deletion virus (Δ491-2,782)</td>
</tr>
<tr>
<td>FAdV-9Δ4-CMV-EGFP</td>
<td>44,627</td>
<td>99.02</td>
<td>Recombinant CMV-EGFP virus</td>
</tr>
<tr>
<td>FAdV-9Δ4-CMV-EGFP-WPRE</td>
<td>45,151</td>
<td>100.2</td>
<td>Recombinant CMV-EGFP-WPRE virus</td>
</tr>
<tr>
<td>FAdV-9Δ4-CAG-EGFP</td>
<td>45,384</td>
<td>100.7</td>
<td>Recombinant CAG-EGFP virus</td>
</tr>
<tr>
<td>FAdV-9Δ4-CAG-EGFP-WPRE</td>
<td>45,908</td>
<td>101.9</td>
<td>Recombinant CAG-EGFP-WPRE virus</td>
</tr>
<tr>
<td>FAdV-9Δ4-EF1α-EGFP</td>
<td>45,190</td>
<td>100.3</td>
<td>Recombinant EF1α-EGFP virus</td>
</tr>
<tr>
<td>FAdV-9Δ4-EF1α-EGFP-WPRE</td>
<td>45,714</td>
<td>101.5</td>
<td>Recombinant EF1α-EGFP-WPRE virus</td>
</tr>
</tbody>
</table>

*Parental virus for recFAdVs
2.4.3 Viral growth curves

One-step growth curves for all viruses were obtained as described by Alexander et al. (1998). Briefly, a total of $1.8 \times 10^6$ CH-SAH cells were seeded in 35 mm dishes and incubated at 37°C with 5% CO₂. The cells were infected with recFAdVs at a multiplicity of infection (MOI) of 5. After adsorption for 1 hour at room temperature, the cells were washed three times with PBS, and fresh DMEM-F12 (5% FBS) was added. Cell culture medium and cells were harvested at 0, 12, 18, 24, 30, 36, 48, 60, and 72 h.p.i. The 0 h.p.i. time-point was defined as 1 hr after the inoculum was added. The medium was removed and frozen at -80°C as the extracellular virus. Cells were washed three times with PBS, 1 ml of medium was placed on the monolayer and the dish frozen at -80°C as the intracellular virus. Extracellular virus was titrated for each time-point. CH-SAH cells were plated in 6-well plates at a density of $1.8 \times 10^6$ cells/well and incubated overnight. Extracellular virus from each time-point was serially diluted ($10^{-1}$-$10^{-7}$), and 100 µl of each aliquot was inoculated in duplicate and allowed to adsorb for 1 hr at room temperature. The inoculum was removed and the monolayer was washed in PBS. Three ml of agar consisting of 0.6% SeaKem LE agarose (Lonza), DMEM-F12 (5% FBS, L-glutamine, and PenStrep) was added to each well, and the plates were incubated at 37°C with 5% CO₂. After five days, 1.5 ml of neutral red (0.015%) was added to each well, and after 24 hrs the plaques were counted.

2.4.4 Detection of EGFP expression

A total of $1.8 \times 10^6$ CH-SAH cells were seeded in 35 mm dishes and incubated at 37°C with 5% CO₂. The cells were infected with recFAdVs at an MOI of 5. Uninfected and FAdV-9Δ4 infected cells were the negative controls, while cells transfected with 6 µg of pEGFP-N1 were the positive control. Cells were collected at 0, 6, 12, 18, 24, 30, 36, and 48 h.p.i. and centrifuged at 5,000 rpm for 5 minutes. Each sample was resuspended in 500 µl of PBS and split.
into two 250 µl aliquots. The first aliquot was stored frozen at -80°C to later be measured by spectrofluorometry. The second aliquot was centrifuged again to wash away FBS. The supernatant was removed and the cell pellet was resuspended in 200 µl of RIPA lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, and 1% sodium deoxycholate). Samples were incubated on ice for 20 minutes, and re-centrifuged at 12,000 rpm at 4°C for 20 minutes. The supernatant was collected and stored at -80°C.

EGFP expression was measured by spectrofluorometry at each time-point. Infected cell samples frozen at -80°C were freeze-thawed three times. The cell debris was centrifuged at 12,000 rpm for 10 minutes at 4°C. Supernatant was transferred to a fresh microcentrifuge tube and the protein concentration was determined at 280 nm using a Nanodrop 2000. All samples were adjusted to a protein concentration of 1 μg/μl. For each sample, 50 µg of protein extract was added in triplicate to a flat-bottomed black 96-well plate and analyzed using a GloMax®-Multi microplate reader to detect EGFP fluorescence using 480 and 528 nm excitation and emission wavelengths, respectively.

2.4.5 Bradford assay and SDS-PAGE

Protein concentration was determined with the BioRad Protein Assay kit as per the manufacturer’s protocol. Briefly, a 1:5 dilution of concentrated dye reagent was made in distilled H₂O. Ten µl of BSA protein standards, ranging in concentration from 100 μg/ml to 1 mg/ml, along with whole cell lysate samples (diluted 1:10) were pipetted into a 96-well plate in triplicate. Two hundred µl of the diluted dye reagent was added to each sample and mixed. After a 5 minute incubation, absorbance was measured at 595 nm in a microplate reader (BioTek Powerwave XS2). A standard curve was generated using the absorbance values of the BSA standards, and the equation of the trend line was used to extrapolate the concentrations of the
unknown cell lysates. Values for each sample were averaged to generate an average total protein concentration, and dilution factor were accounted for. All samples were diluted to a final concentration of 0.67 μg/μl in distilled H₂O.

Proteins were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Ten percent acrylamide gels were prepared according to recipes obtained from the Roche Lab FAQs Handbook (4th Ed). For all experiments, 15 μl of each cell lysate (0.67 μg/μl) was mixed with 4 μl of 4x SDS-PAGE loading buffer (supplemented with 2-mercaptoethanol). Samples were incubated at 95°C for 10 minutes prior to being loaded in the gels. A total of 10 μg was loaded per sample into individual lanes, as well as 5 μl of protein ladder (Precision Plus Protein Dual Colour, BioRad). Once all samples were loaded, gels were run at 100 V for approximately 1.5 hours in running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS, pH 8.3).

2.4.6 Western immunoblot

Following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes in a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) as per the manufacturer’s protocol. Samples were run at 100 V for 1 hour in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH 8.3). After transfer, membranes were rinsed in Tris-buffered saline supplemented with 0.1% Tween 20 (TBS-T) and blocked with 5% skim milk (in TBS-T) for 1 hour at room temperature with agitation. Primary antibody was added to the blocking solution and membranes were incubated overnight at 4°C. To probe for EGFP, primary monoclonal mouse anti-GFP antibody (Molecular Probes) was used at a dilution of 1:1,000. To probe for actin, primary polyclonal goat anti-actin antibody (Santa Cruz Biotechnology) was used at a dilution of 1:1,000. The following day, all blots were washed three times in TBS-T for 10 minutes each with agitation. The blots were then incubated in secondary antibody diluted in
blocking solution for 1 hour with agitation. The following polyclonal secondary antibodies conjugated with horseradish peroxidase (HRP) were used: goat anti-mouse (Invitrogen, at 1:5,000 dilution), and donkey anti-goat (Jackson, at 1:10,000 dilution). Blots were then washed three times in TBS-T, and incubated in Western Lightning Plus-ECL reagent (Perkin Elmer Inc) for five minutes before being developed using a ChemiDoc XRS (BioRad).

2.5 Statistical analyses

Statistical analyses were performed with GraphPad Prism 6.0 (La Jolla, CA). The significance of expression by constructs in comparison to pCMV-EGFP-Luc after normalization at each individual time-point was determined by *t*-test. Furthermore, the *t*-test was used to determine the significance of expression by recFAdVs in comparison to FAdV-9Δ4-CMV-EGFP measured by fluorometry, and between promoter constructs with and without WPRE, at each individual time-point. Results were deemed significant at 0.01 < P < 0.05, very significant at 0.001 < P < 0.01, and extremely significant at 0.0001 < P < 0.001.
Chapter 3. Results

3.1 Transient expression of EGFP in transfected CH-SAHC cells

3.1.1 Generation of dual-expression constructs

A dual-expression system was created using EGFP under different promoters and firefly luciferase (expressed under the SV40 promoter) to compare the strength of different promoter and enhancer elements on EGFP expression in vitro. The commercial plasmid pCI-Neo (Promega), containing the CMV enhancer, promoter, and intron, was the backbone for the dual-expression system. The CMV promoter (944 bp) was subsequently removed using RE digestion with SpeI and EcoRI. After gel purification, both CAG (1,701 bp) and EF1α (1,507 bp) promoters were directionally sub-cloned into the pCI-Neo backbone using SpeI and EcoRI. Ligated product was transformed into competent bacterial cells and colonies were selected and screened by RE digestion (results not shown) and confirmed by sequencing. This process was repeated for both the β-actin (285 bp) and L2R promoters (120 bp). The RE sites for SpeI and EcoRI were inserted into primers and both promoters were PCR amplified from plasmid DNA.

A 730 bp fragment corresponding to EGFP was PCR amplified with primers containing EcoRI and NotI sites. The PCR product was directionally cloned into each promoter plasmid, transformed into competent bacterial cells and confirmed by both PCR (data not shown) and sequencing. Finally, firefly luciferase (1,712 bp) was PCR amplified with primers containing the RE sites AvrII and BstBI. Plasmid DNA, containing EGFP under the control of each promoter, was then digested with AvrII and BstBI to remove the neomycin resistance gene, and luciferase was directionally cloned in its place. The presence of luciferase in each plasmid was confirmed by PCR (results not shown) and sequencing. The following dual-expression plasmids were obtained: pCMV-EGFP-Luc, pCAG-EGFP-Luc, pEF1α-EGFP-Luc, pβactin-EGFP-Luc, and
pL2R-EGFP-Luc (Figure 3.1). Five additional plasmids were generated to include the enhancer element WPRE. A 543 bp fragment corresponding to WPRE was PCR amplified with primers containing a NotI site. Each dual-expression plasmid was linearized with NotI and the WPRE element was non-directionally cloned into each. Proper orientation was screened by PCR (data not shown) and plasmids were confirmed by sequencing, resulting in the dual-expression plasmids: pCMV-EGFP-WPRE-Luc, pCAG-EGFP-WPRE-Luc, pEF1α-EGFP-WPRE-Luc, pβactin-EGFP-WPRE-Luc, and pL2R-EGFP-WPRE-Luc (Figure 3.1).

3.1.2 Expression patterns of promoters observed by fluorescence microscopy

CH-SAH cells were transfected with the dual-expression constructs to follow EGFP expression patterns over 72 h.p.t. by fluorescence microscopy (Figure 3.2). The earliest expression of EGFP was noted at 12 h.p.t. by CMV, CMV-WPRE, CAG, and CAG-WPRE plasmids, with expression levels increasing over-time. Both EF1α and EF1α-WPRE constructs began expressing EGFP between 24 and 36 h.p.t. which then remained steady over-time. Constructs containing the β-actin or L2R promoters showed the weakest expression of EGFP in CH-SAH cells, with expression observed between 36 and 72 h.p.t., and between 60 and 72 h.p.t. respectively. All constructs were compared to mock transfected cells (negative control) and a positive control of pEGFP-N1 transfected cells (data not shown).

3.1.3 Normalized expression of promoter constructs

Dual-expression constructs were transfected into CH-SAH cells and transgene expression was measured over 72 h.p.t. The fluorescence of EGFP was measured by fluorometry at each time-point, while the luminescence from luciferase was measured using a Peirce Firefly Luciferase Glow Assay kit. To better analyse the expression of EGFP driven by each promoter/enhancer element, and to minimize sample-to-sample variation, the
A)

pCMV-EGFP-Luc (7003 bp)

5'  | 3'
---|---
CMV | EGFP

EcoRI

NotI

B)

pCAG-EGFP-Luc (7760 bp)

5'  | 3'
---|---
CAG | EGFP

EF1α

βactin

pL2R-EGFP-Luc (6179 bp)

5'  | 3'
---|---
CAG | EGFP

EF1α

βactin

pCMV-EGFP-WPRE-Luc (7546 bp)

5'  | 3'
---|---
CMV | EGFP

WPRE

NotI

pCAG-EGFP-WPRE-Luc (8295 bp)

5'  | 3'
---|---
CAG | EGFP

WPRE

pEF1α-EGFP-WPRE-Luc (8101 bp)

5'  | 3'
---|---
EF1α | EGFP

WPRE

pβactin-EGFP-WPRE-Luc (6879 bp)

5'  | 3'
---|---
βactin | EGFP

WPRE

pL2R-EGFP-WPRE-Luc (6714 bp)

5'  | 3'
---|---
L2R | EGFP

WPRE
Figure 3.1. Schematic representation of EGFP/luciferase dual-expression plasmids. (A) EGFP was PCR amplified from pEGFP-N1 and cloned into the multiple cloning site of pCI-Neo. The neomycin resistance (NeoR) gene from pCI-Neo was removed by restriction enzyme digestion. Firefly luciferase was PCR amplified from pGL-4.17 and cloned under the SV40 promoter, resulting in a dual-expression plasmid. (B) The plasmid pCMV-EGFP-Luc was digested with SpeI and EcoRI to clone in promoters of interest, resulting in five vectors controlling EGFP under different promoters and luciferase under the SV40 promoter: pCMV-EGFP-Luc, pCAG-EGFP-Luc, pEF1α-EGFP-Luc, pβ-actin-EGFP-Luc, and pL2R-EGFP-Luc. Five additional plasmids were made by non-directionally cloning the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) into each dual-expression plasmid at the NotI site, resulting in the constructs: pCMV-EGFP-WPRE-Luc, pCAG-EGFP-WPRE-Luc, pEF1α-EGFP-WPRE-Luc, pβ-actin-EGFP-WPRE-Luc, and pL2R-EGFP-WPRE-Luc.
Figure 3.2. Comparison of EGFP expression with fluorescence microscopy. CH-SAH cells were seeded in 35 mm plates (1.8x10⁶ cells/dish) and transfected with 2 μg of plasmid DNA. Fluorescence of EGFP was measured by microscopy at 12, 24, 36, 48, 60, and 72 hours post-transfection. A mock transfection was the negative control while pEGFP-N1 was the positive control. Abbreviations of the promoters and the presence of WPRE (-W) is given to the left.
expression of luciferase from each sample was used to normalize the results (Schagat et al., 2007). The data were analysed by calculating the fold change of each construct, at each specific time post-transfection, in relation to the CMV promoter (Appendix Table 1). Values higher than 1.0 suggest expression better than the CMV promoter, and values less than 1.0 suggest lower expression compared to the CMV promoter. The normalized expression of each construct from 12-72 h.p.t. is shown in Figure 3.3. At 12 h.p.t. a significantly lower level of expression was observed by EF1α, EF1α-WPRE, β-actin-WPRE, and L2R-WPRE constructs compared to CMV. CMV-WPRE and CAG constructs had increased expression at 12 h.p.t. however this was not significantly different from the CMV alone. Greater differences in expression patterns were seen between 24-72 h.p.t. In general, all constructs showed decreased activity over-time compared to the CMV control. At both 12 and 24 h.p.t. the CMV-WPRE construct showed an increased fold change of 1.047 over CMV only, though the differences were not significant. Between 36 and 60 h.p.t. the CMV-WPRE showed a slightly decreased fold change ranging from 0.851 to 0.922. A significant decrease in expression was detected at 72 h.p.t., when CMV-WPRE showed a lower activity level of 64% compared to the CMV control, and this difference was significant. For each of the remaining constructs, expression was significantly below that of CMV at all time-points where P <0.05. Constructs containing the CAG and CAG-WPRE cassettes consistently expressed at a range of approximately 50% and 40%, respectively, compared to CMV from 24 h.p.t. Similarly, constructs containing the EF1α and EF1α-WPRE cassettes were found to have a decreased activity level compared to CMV, expressing at a range of approximately 20%. The remaining constructs, containing the β-actin and L2R promoters, had activity levels less than 10% that of CMV.
Figure 3.3. Normalized EGFP expression over-time. CH-SA cells were seeded in 35 mm plates (1.8x10^6 cells/plate) and transfected with 2 μg of plasmid DNA. At each time-point, transfected cells were washed, trypsinized, and resuspended in PBS. After three freeze-thaw cycles, samples were centrifuged and the protein concentration of the collected supernatant was measured with a nanodrop. Fluorescence of EGFP was measured in a microplate reader at 480 and 528 nm excitation and emission wavelengths, respectively. Luminescence of luciferase was measured using a Pierce Firefly Luciferase Glow Assay kit. 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 significance of expression compared to pCMV-EGFP-Luc, indicated by an asterisks (*), where P <0.05.
3.2 Generation of recombinant FAdV-9Δ4s

3.2.1 Cloning of new intermediate constructs

To further analyze promoter activity and the activity of WPRE in the context of FAdV replication, recombinant FAdVs containing the most efficient EGFP expression cassettes were generated following a method modified from Corredor and Nagy (2010b). A 477 bp fragment (VF1) left of the FAdV-9Δ4 deletion site was PCR amplified and directionally cloned into pCMV-EGFP-Luc, pCMV-EGFP-WPRE-Luc, pCAG-EGFP-Luc, pCAG-EGFP-WPRE-Luc, pEF1α-EGFP-Luc, and pEF1α-EGFP-WPRE-Luc. Subsequently, a 1609 bp fragment (VF2) right of the FAdV-9Δ4 deletion site was PCR amplified and cloned into each plasmid, resulting in the intermediate plasmids: pHMR-CMV-EGFP, pHMR-CMV-EGFP-WPRE, pHMR-CAG-EGFP, pHMR-CAG-EGFP-WPRE, pHMR- EF1α-EGFP and pHMR- EF1α-EGFP-WPRE (Figure 3.4). Successful cloning was determined by PCR screening and sequencing.

Intermediate constructs were subjected to PCR or RE digestion with BglII/BamHI to isolate promoter EGFP cassettes flanked by viral DNA fragments for homologous recombination with pFAdV-9Δ4 (FAdmid). The resulting FAdmids were generated upon homologous recombination: pFAdV-9Δ4-CMV-EGFP, pFAdV-9Δ4-CMV-EGFP-WPRE, pFAdV-9Δ4-CAG-EGFP, pFAdV-9Δ4-CAG-EGFP-WPRE, pFAdV-9Δ4-EF1α-EGFP, and pFAdV-9Δ4-EF1α-EGFP-WPRE in E. coli BJ5183 cells. Successful recombination was determined by sequencing and NotI digest screening (Figure 3.5). Digestion of pFAdV-9Δ4 resulted in a 4 kb, a 5.1 kb, a 13.7 kb, and a 23.8 kb fragment. FAdmids containing the CMV cassettes were identified by a diagnostic 2.2 kb band corresponding to the CMV promoter plus the EGFP gene and SV40 poly(A) tail. FAdmids containing the CAG cassettes were identified by a diagnostic 2.9 kb band, and EF1α by a diagnostic 2.7 kb band. Additional bands at 550 bp were present in FAdmids.
Figure 3.4. Schematic representation of intermediate constructs used to generate recFAdVs. (A) FAdV-9 DNA flanking the Δ4 deletion site (VF1 and VF2) was PCR amplified and directionally cloned into the dual-expression plasmid pCMV-EGFP-Luc. The resulting construct, pHM-9Δ4-CMV-EGFP, was used downstream to generate the recombinant virus FAdV-9Δ4-CMV-EGFP. (B) Five additional intermediate constructs were generated, resulting in the plasmids: pHMR-CAG-EGFP, pHMR-EF1α-EGFP, pHMR-CMV-EGFP-WPRE, pHMR-CAG-EGFP-WPRE, and pHMR-EF1α-EGFP-WPRE.
Figure 3.5. Agarose gel electrophoresis of NotI digested FAdmids. FAdmids derived from homologous recombination between pFAdV-9Δ4 and pHMR plasmids in E. coli BJ5183 cells were digested with NotI and the resulting banding patterns were screened in agarose gel. Digested pFAdV-9Δ4 has an expected banding pattern of 4 kb, 5.1 kb, 13.7 kb, and 23.8 kb. Recombinant FAdmids with an EGFP expression cassette contained an additional NotI site for screening. Diagnostic bands corresponding to each cassette were as follows: CMV-EGFP= 2.2 kb, CMV-EGFP-WPRE= 2.2 kb and 0.55 kb, CAG-EGFP= 2.9 kb, CAG-EGFP-WPRE= 0.55 kb, EF1α-EGFP= 2.7 kb, and EF1α-EGFP-WPRE= 2.7 kb and 0.55 kb. White arrows point to the diagnostic bands.
containing the WPRE element. These FAdmids were transfected into CH-SAH cells to generate the corresponding viruses.

### 3.2.2 Viral growth kinetics

Viral growth kinetics were compared among the six recombinant viruses and the reference FAdV-9Δ4 to determine whether the addition of any promoter EGFP cassette affected virus replication and growth. The virus titer and growth of all recFAdVs appeared to follow a similar pattern to FAdV-9Δ4, except that FAdV-9Δ4-CAG-EGFP and FAdV-9Δ4-EF1α-EGFP had one half log lower titer than the reference starting at 24 h.p.i. and continuous to 72 h.p.i. (Figure 3.6). Viruses yielded the following titers: FAdV-9Δ4 = 2.4x10^8 pfu/ml, FAdV-9Δ4-CMV-EGFP = 4.8x10^8 pfu/ml, FAdV-9Δ4-CAG-EGFP = 8.3x10^7 pfu/ml, FAdV-9Δ4-EF1α-EGFP = 8.2x10^7 pfu/ml, FAdV-9Δ4-CMV-EGFP-WPRE = 3.1x10^8 pfu/ml, FAdV-9Δ4-CAG-EGFP-WPRE = 3.0x10^8 pfu/ml, and FAdV-9Δ4-EF1α-EGFP = 3.7x10^8 pfu/ml. The appearance of CPE (Figure 3.7) and plaque morphology (not shown) of recFAdVs were similar to that of FAdV-9Δ4 in that the cells rounded and detached by 5 - 7 d.p.i. All recFAdVs expressed EGFP as seen by fluorescence microscopy.

### 3.3 Promoter activity during infection

#### 3.3.1 Measuring EGFP by spectrofluorometry

EGFP expression by recFAdVs was measured between 0 - 48 h.p.i. in CH-SAH cells. Based on both spectrofluorometry and fluorescence microscopy, expression of EGFP from all recombinant viruses was low until 12 h.p.i. Strong expression of EGFP was noted between 12-30 h.p.i. but it declined after 36-48 h.p.i. Maximum fluorescence readings were measured at 30 h.p.i. for all recFAdVs (Figure 3.8). Cells infected with FAdV-9Δ4-CAG-EGFP showed the greatest increase in EGFP expression compared to FAdV-9Δ4-CMV-EGFP at all time-points,
Figure 3.6. Viral growth curves. One-step growth curve for each recombinant FAdV was determined in CH-SAH cells. Cells were seeded in 35 mm plates (1.8x10^6 cells/plate) and infected at an MOI of 5. Both intracellular and extracellular virus was collected between 0-72 h.p.i. One-step growth curves of the extracellular viruses were done twice and each sample was titrated in duplicate by plaque assay.
Figure 3.7. Cytopathic effect of recombinant FAdV is similar to FAdV-9Δ4. CH-SAH cells were plated in 35 mm plates (1.8x10^6 cells/plate) and infected at an MOI of 5. Cytopathic effect of recombinant viruses was compared to the parental control FAdV-9Δ4. All recombinant viruses (data not shown) had similar CPE to FAdV-9Δ4, evidenced by cell rounding and detachment using a bright-field microscope. Fluorescence of EGFP by recFAdVs, for example FAdV-9Δ4-CAG-EGFP-WPRE, was observed by fluorescence microscopy.
Figure 3.8. Time course of EGFP expression in CH-SAH cells. CH-SAH cells were seeded in 35 mm plates (1.8x10^6 cells/plate) and infected with recFAdV-9s at an MOI of 5. At each time-point, infected cells were collected, washed, and resuspended in PBS. After three freeze-thaw cycles, samples were centrifuged and the protein concentration of the collected supernatant was measured with a nanodrop. The absolute fluorescence of EGFP from 50 µg of whole cell lysate was measured with a microplate reader at 480 and 528 nm excitation and emission wavelengths, respectively. Uninfected (mock) or FAdV-9Δ4 infected cells did not show any fluorescence. The t-test was used to determine the significance of expression in comparison to pCMV-EGFP-Luc as indicated by an asterisks (*), where significant (*, 0.01 < P < 0.05), very significant (**, 0.001 < P < 0.01), and extremely significant (***, 0.0001 < P < 0.001) are shown.
ranging from 12 (48 h.p.i.) to 29-fold (24 h.p.i) greater in expression. Expression of EGFP by FAdV-9Δ4-CAG-EGFP was significantly higher \((P < 0.05)\) at all time-points after 12 h.p.i. when compared to FAdV-9Δ4-CMV-EGFP. FAdV-9Δ4-EF1α-EGFP infected cells also had increased expression compared to FAdV-9Δ4-CMV-EGFP at almost all time-points, with a slightly lower range of 2 (12 h.p.i) to 20-fold (24 h.p.i) higher in expression. Expression of EGFP by FAdV-9Δ4- EF1α-EGFP was also significantly higher \((P < 0.05)\) at all time-points after 24 h.p.i. when compared to FAdV-9Δ4-CMV-EGFP. Viruses that contained a WPRE element expressed EGFP at a lower level when compared to their promoter only counterparts. FAdV-9Δ4-CMV-EGFP-WPRE was the “worst performing” virus, with 2.4 (48 h.p.i.) to 17-fold (36 h.p.i.) lower expression level compared to FAdV-9Δ4-CMV-EGFP. This result was found to be significant at 18, 30, 36, and 48 h.p.i. Although both FAdV-9Δ4-CAG-EGFP-WPRE and FAdV-9Δ4-EF1α-EGFP-WPRE performed worse than their promoter only counterparts, both viruses on average expressed EGFP at a higher level than FAdV-9Δ4-CMV-EGFP. FAdV-9Δ4-CAG-EGFP-WPRE showed increased expression ranging from 1.3 (48 h.p.i.) to 4.3-fold (24 h.p.i.), with significant increases in expression at 18 and 24 h.p.i. FAdV-9Δ4-EF1α-EGFP-WPRE expression ranged from a 2.5-fold decrease (12 h.p.i.) to 3.6-fold increase (24 h.p.i.), with significant increases in expression at 24 and 30 h.p.i.

### 3.3.2 Expression of EGFP as evaluated by Western blot

In addition to spectrofluorometry, viral EGFP expression was also examined by Western immunoblot. Whole cell lysates were collected from infected cells at 0, 6, 12, 18, 24, 30, 36, and 48 h.p.i. At each time-point, 10 µg of total protein from each lysate was separated by SDS-PAGE, blotted, and the blot was probed with anti-EGFP antibody (Figure 3.9). The molecular mass of EGFP is 27 kDa (Takebe et al., 2002). At early time-points of 0 and 6 h.p.i., no EGFP
signal was detected from any recFAdVs. By 12 h.p.i., a band corresponding to EGFP was
detected in lysate collected from FAdV-9Δ4-CAG-EGFP infected cells, which was detected up
until 48 h.p.i. Cell lysates collected from FAdV-9Δ4-EF1α-EGFP, FAdV-9Δ4-CAG-EGFP-
WPRE, and FAdV-9Δ4-EF1α-EGFP-WPRE also produced a detectable EGFP band from 18-36
h.p.i. In contrast, no EGFP bands were seen for FAdV-9Δ4-CMV-EGFP and FAdV-9Δ4-CMV-
EGFP-WPRE infected lysates at any time-points. The negative controls, FAdV-9Δ4 infected
lysate and mock infected lysate, showed no signal for EGFP throughout the time-course.
Transfected CH-SAH cells containing the EGFP positive plasmid under the CMV promoter,
pEGFP-N1, gave a positive signal from 12 h.p.t. onwards. At each time-point blots were probed
with an anti-actin antibody to show relative levels of equal loading of all samples. Actin levels
appeared similar at each time-point, suggesting equal loading, however at 48 h.p.i. actin was not
detected in virus infected lysates. At all time-points the strongest expression was detected for
FAdV-9Δ4-CAG-EGFP, followed by FAdV-9Δ4-EF1α-EGFP, reflecting the results based on
EGFP fluorescence.
Figure 3.9. Western immunoblot of EGFP production over-time. EGFP expression by recFAdV-9s in CH-SA H cells was compared over 48 h.p.i., along with FAdV-9Δ4 (negative control), uninfected (negative control), and pEGFP-N1 transfected CH-SA H cells (positive control). CH-SA H cells were seeded in 35 mm plates (1.8x10^6 cells/dish) and infected with recFAdV-9s at an MOI of 5. At each time-point whole cell lysates were collected and protein concentrations were determined by Bradford assay. Ten µg of each sample was loaded into two gels, one to be probed with anti-EGFP antibody (1:1,000) followed by a secondary anti-mouse HRP conjugated antibody (1:5,000), the other gel was probed with anti-actin antibody (1:1,000) followed by a secondary anti-goat HRP conjugated antibody (1:10,000). All bands appeared at the expected size (EGFP = 27 kDa and actin = 42 kDa) as determined from a molecular weight marker (not shown).
Chapter 4. Discussion

Members of the family *Adenoviridae* have been extensively studied and characterized for their application as recombinant vaccine and gene therapy vectors. Specifically, FAdV based vectors replacing both left (Corredor and Nagy, 2010b) and right end (Sheppard *et al*., 1998; Johnson *et al*., 2000; Ojkić and Nagy, 2001; Francois *et al*., 2003; 2004) non-essential genomic regions can accommodate large transgenes, making them attractive vectors. Current research in the Nagy laboratory has focused on exploiting a 2.4 kb non-essential region of FAdV-9 for transgene insertion, leading to the development of the deletion virus FAdV-9Δ4 (Corredor and Nagy, 2010a). Recombinant FAdV-9 viruses have been generated by inserting foreign genes into this non-essential region, such as EGFP and HA, and *in vitro* studies have confirmed that these viruses exhibit growth characteristics and CPE similar to wtFAdV-9 (Corredor and Nagy, 2010b). Inoculation of chickens with recFAdV-9 does stimulate a host immune response to the foreign gene (Corredor and Nagy, 2011), and this response is protective (Gay *et al*., 2015), however there is room for further improvement. The aim of this study was to increase foreign gene expression and protein production from FAdV-9Δ4 based vectors by testing and comparing the effectiveness of a variety of constitutively active promoters and one regulatory element on transgene expression, in comparison to the currently used CMV promoter.

Prior to investigating promoter activity in recFAdVs, the first objective of this study was to compare and contrast promoter strength in a chicken hepatoma cell line to determine which promoter constitutively expressed EGFP the best. In the current system for developing recFAdV-9Δ4 viruses, the transgenes are expressed under the CMV promoter (Corredor and Nagy, 2010b). Interestingly, previous studies in mammalian cell lines reported that CMV induced expression varies drastically from cell type to cell type (Qin *et al*., 2010). Additionally, transgene silencing
due to methylation of the CMV promoter in vivo is commonly reported in mammalian systems, which has led to the migration away from using the CMV promoter (Brooks et al., 2004; Mehta et al., 2009). It can be speculated that the CMV promoter may not be the ideal promoter for recFAdV-9 vaccines, there is a need for better alternatives. Previous research in our laboratory has shown that the CMV promoter induces EGFP expression in a variety of avian cells, including CH-SAH, quail fibroblast (QT-35), and primary chicken embryo liver (CELi) cells, as evidenced by fluorescence microscopy (Corredor and Nagy, 2010b). Additionally, Seo et al. (2010) demonstrated not only that both the CMV and CAG promoters induce EGFP expression in electroporated chicken embryonic fibroblast (DF-1) cells, but also that the CAG promoter induced stronger levels of expression.

The strength of four promoters (CAG, EF1α, β-actin, and L2R) was compared to each other and that of CMV in CH-SAH cells, in addition to measuring the effects of WPRE on expression. To achieve this, a novel dual promoter system was generated using the commercial plasmid pCI-Neo. Traditionally, genetic reporter systems are used to study eukaryotic gene expression. Moreover, dual promoter systems, where two independent reporter genes are expressed under individual promoters in the same plasmid, are commonly used to improve experimental accuracy when an internal control is needed to provide a baseline response for normalization (Sherf et al., 1996; McNabb et al., 2005; Shimizu and Shimizu, 2013). In this study, the EGFP coding region was cloned into the multiple cloning site of pCI-Neo, downstream of each promoter being tested, as its expression can be easily measured by microscopy and fluorometry. Firefly luciferase was PCR amplified and cloned into pCI-Neo under the SV40 promoter, providing a measurable reporter gene to normalize between groups. Previous studies report that the SV40 promoter induces very low levels of expression in
transfected CH-SAH cells (Scholz et al., 1993). When compared to fluorescent reporters, luminescent proteins are 10-1000 fold more sensitive (Wood, 2007), thus I postulated that firefly luciferase (rather than another fluorescent reporter gene) would overcome the relatively weak activity of the SV40 promoter and provide a measurable value to normalize with.

Fluorescence microscopy confirmed the expression of EGFP in transfected CH-SAH cells by all dual-expression constructs (Figure 3.2). Transfected cells were harvested at various time points and both raw fluorescence and luminescence were measured as described in Chapter 2. Promoter induced expression of EGFP in each construct was normalized using luciferase and in relation to the CMV construct (Figure 3.3 and Appendix Table 1). Normalized data suggest that the CMV promoter (including the native CMV enhancer and intron) induces optimal expression in transfected CH-SAH cells. Significantly lower levels in expression relative to CMV were observed between 24-72 h.p.t. by the CAG, EF1α, β-actin and L2R constructs. Therefore, promoter strength in transfected CH-SAH cells can be described as follows: CMV>CAG>EF1α>β-actin>L2R. Further, the presence of WPRE seemed to have a negative effect on expression, which was evident by the difference in expression among constructs containing the same promoters with or without WPRE.

Research on expression cassettes in avian cell lines is rather limited, yet some of my findings contradict what has been previously described in the literature. I had anticipated that the CAG promoter would induce the strongest levels of EGFP expression. When Seo et al. (2010) compared CMV and CAG activity in DF-1 cells, they found that CAG increased EGFP expression 1.6-fold as measured by flow cytometry. Furthermore, when Jiang et al. (2007) compared CMV and CAG induced expression of HA they reported that CAG induced expression led to significantly higher level of HA protein in transfected chicken embryo fibroblasts (CEL).
as measured by Western immunoblot at 48 h.p.t. A simple explanation for my findings could be that CAG performs better than CMV in fibroblast cells, but not in hepatoma cells. A systematic comparison of constitutive promoters in avian cell lines is yet to be done, but similar studies testing a variety of cell lines and species have been reported in mammalian systems. For example, Qin et al. (2010) tested eight commonly used constitutive promoters (including CMV, CAG, and EF1α) in multiple cell lines derived from several species using lentivirus expression vectors containing GFP. With flow cytometry it was reported that although most promoters have consistent activity levels among cell types (including CAG and EF1α) the CMV activity varies considerably, especially in cell lines derived from fibroblasts. Therefore applying the findings of Qin et al. (2010), the differences obtained between my study and that of Seo et al. (2010) and Jiang et al. (2007) may be due to the cell line, thus further study using my dual-expression system is warranted in chicken fibroblast and other cells. Another explanation for the differences between Seo et al. (2010) and my data could be due to the experimental conditions. In my study I collected and measured transient expression in transfected cell lysates between 12-72 h.p.t. Meanwhile, Seo et al. (2010) selected for stable electroporated and transformed cells using G418 and then measured EGFP expression by flow cytometry after 90-days of sub-culture. It is unclear whether the DNA of Seo et al. integrated into the cellular genome after selection over 90-days. It is probable that the CAG and CMV promoter cassettes became incorporated into the DF-1 cell genome, as this is a hallmark of stable transfection. If this is the case, the results of Seo et al. (2010) could be related to the site of integration. Transient expression and stable expression are two very different model systems, and therefore not entirely comparable. For arguments sake and putting systems aside, it may be that the CAG promoter induces stronger long-term expression in avian cells, and that the CMV promoter induces stronger immediate-early expression. Therefore,
further comparison of the CMV and CAG promoters is warranted in both CH-SAHL and DF-1 cells.

The CMV, CAG, and EF1α promoters were all studied as they are commonly used in mammalian expression systems due to their ability to mediate high levels of gene expression (Qin et al., 2010; Chen et al., 2011, Damdindorj et al., 2014). Prior to this study, EF1α promoter activity in avian cell lines was unknown. Based on my results it can be concluded that the EF1α promoter induces EGFP expression in transfected CH-SAHL cells, although at significantly lower levels than CMV promoter. In eukaryotic cells, EF1α is considered to be one of the most abundant proteins supporting GTP-dependent binding of an aminoacyl-tRNA to ribosomes, as well as a linker between protein translation and the cytoskeleton (Uetsuki et al., 1989; Liu et al., 2002). While the EF1α promoter studied in this work was derived from human chromosomal DNA, it appears that its promoter activity was independent of host cell type, which is most likely due to the highly conserved nature of the EF1α locus (Uetsuki et al., 1989). Further analysis of the EF1α promoter is necessary as it may provide a more consistent expression pattern in a variety of cell types during recFAdV-9 infection.

The chicken β-actin and L2R promoters were tested as they represented avian derived native and virus associated promoters, respectively. Both of these promoters induced very low levels of expression in comparison to CMV. The β isoform of actin is abundant in cells as it is a major component of the cytoskeleton. Initial studies of this promoter found that it induced strong expression in a variety of cell types, specifically in comparison to the RSV and SV40 promoters, which ultimately led to its use in expression vectors and more importantly as a constituent of the CAG promoter (Fregien and Davidson, 1986; Miyazaki et al., 1989; Niwa et al., 1991). In my study, the β-actin promoter was PCR amplified from the CAG promoter cassette as a 285 bp
product, however the β-actin intron encoded downstream of the β-actin promoter was excluded, as recFAdVs have a limited space capacity for transgene cassettes and therefore a small yet strong promoter would be valuable when developing a multivalent recFAdV-9. The weak expression by the β-actin construct may be due to its lack of intron. Introns enhance the rate of RNA polyadenylation and nuclear export (Huang and Gorman, 1990), and multiple studies demonstrated that the inclusion of intron in both plasmid and viral vectors increases transgene expression (Xu et al., 2001; Schiedner et al., 2002; White et al., 2008). In this study, the expression cassettes including introns downstream of the promoter element (CMV, CAG, and EF1α) induced the highest levels of EGFP expression. Therefore, further study of the chicken β-actin promoter in avian cell lines should include the native intron, which is also present in the CAG promoter.

Unlike native chicken β-actin, the L2R promoter was derived from a virus. Transcriptional analysis of FPV identified a putative early/late promoter (L2R) similar to the well-characterized vaccinia virus (VV) early promoter, which was capable of driving transient expression of the Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) gene (Zantinge et al., 1996). As a member of the family Poxviridae, FPV replication occurs in the cytoplasm, and therefore the virus encodes transcription factors and enzymes required to initiate the transcription of the early genes. Specifically, poxvirus transcription requires a viral encoded DNA-dependent RNA polymerase (Kates and McAuslan, 1967; Broyles, 2003). It is likely that the L2R promoter was unable to induce EGFP expression because the host cell DNA-dependent RNA polymerase did not recognize the viral promoter initiation sites. To confirm this hypothesis, I suggest transfecting pL2R-EGFP-Luc into FPV infected CH-SAH cells and comparing EGFP expression again, as FPV encoded transcription factors and polymerase should
stimulate L2R induced EGFP expression. The SV40 promoter, which induces luciferase expression, may outcompete both the chicken β-actin and L2R promoters for cellular transcription factors. Without determination of the exact transcription factors utilized by the promoters tested in my study, I could evaluate this hypothesis by flow cytometry and normalizing to plasmid copy number instead of a luciferase reference. To do so, I would have to generate constructs for all five promoters expressing EGFP in a pGEM-T Easy backbone, in addition to a new construct with the SV40 promoter, and analyze promoter strength as described by Seo et al. (2010) in the absence of the SV40-luciferase cassette.

The effect of WPRE in each promoter expression cassette was evaluated in transfected CH-SAHA cells. Post-transcriptional regulatory elements, like that of woodchuck hepatitis virus, are cis-acting RNA elements that increase cytoplasmic mRNA levels by promoting both mRNA export and stability. The inclusion of these elements in both viral and plasmid based vectors has led to increased protein production (2-50 fold) and in turn increased immune response both in vitro and in vivo (Zufferey et al., 1999; Xu et al., 2003; Sun et al., 2009; Chen et al., 2013), which therefore warranted testing in CH-SAHA cells and recFAdVs. Results from my study suggest that the presence of WPRE seemed to have a slight negative effect on normalized EGFP expression in CH-SAHA cells. There is limited study of WPRE activity in avian cell lines, however two previous studies have reported opposite findings to mine. First, as mentioned above Seo et al. (2010) compared CMV and CAG driven expression of EGFP in DF-1 cells, but also analyzed the effects of WPRE and other transcription regulatory elements. They reported that WPRE had a significant enhancing effect on protein as expression measured by flow cytometry for both CMV (1.7-fold increase) and CAG (1.2-fold increase) constructs. These results are supported by the findings of Li et al. (2013), who showed a 2-fold increase in VP2 protein level.
by WPRE containing plasmids in transfected DF-1 cells measured by Western immunoblot. Therefore, prior to my study the consensus was that WPRE stimulates increased mRNA export into the cytoplasm and in turn improves protein expression in avian cells. The effect WPRE has on transgene expression is position dependent, where the WPRE sequence must be inserted between the stop codon of the gene of interest and the polyadenylation signal, in proper orientation (5’ to 3’) (Zufferey et al., 1999). When comparing vector design between Seo et al. (2010), Li et al. (2013), and myself, it is clear that all three studies cloned WPRE in the proper orientation downstream of the transgene stop codon, thus ruling out the difference due to positional effect. It is important to note that like CAG promoter activity, the effect of WPRE in CH-SAHellobs could in fact be cell line specific, as has been found in many mammalian cell lines (Ramezani et al., 2000; Kraunus et al., 2004; Klein et al., 2006). Further, the positive effect of WPRE on expression has been linked to CRM-1 (exportin) dependent nuclear export pathway (Popa et al., 2002). In which case the differences between CH-SAHellobs and DF-1 cells could be due to varying levels of CRM-1 between the cell lines, nevertheless further experimentation would be needed to determine this.

After analysing transient promoter activity in transfected CH-SAHellobs, the next step was to characterize promoter activity in the context of viral infection. Based upon the transient expression, the CMV promoter induced the highest level of expression, followed by the CAG and EF1α promoters. All three promoters and the WPRE element were compared for their ability to promote the expression of EGFP in recFAdVs during virus replication. Characterization and manipulation of the FAdV-9 genome were done via the FAdmid system, in which a plasmid containing the entire FAdV-9 genome (pPacFAdV9, pFAdV9, or FAdmid) is manipulated in bacteria by homologous recombination (Ojkić and Nagy, 2001). Early generation of recombinant
adenoviruses, specifically mammalian ones, relied on direct manipulation of viral DNA by restriction enzymes. In this method transgenes were ligated directly into the viral genome, but the ability to do so was limited by the number of unique enzyme sites as well as the low efficiency of large DNA ligations and repeated rounds of plaque purification (Miyake et al., 1996; Mizuguchi and Kay, 1998; Gao et al., 2003). Next-generation systems, like the FAdmid system, circumvent these limitations by relying on homologous recombination between large plasmids containing the majority of the viral coding regions and a shuttle plasmid containing a target expression cassette flanked by viral DNA in either bacteria or mammalian cell lines (Chartier et al., 1996; Nakano et al., 2005; Corredor and Nagy, 2010b; Miravet et al., 2014). These systems are not without their own limitations, as efficiency, screening, and contamination with wild-type virus still remain an issue.

I first attempted to generate recFAdVs as reported by Corredor and Nagy (2010b). Briefly, this method was adapted from Ojkić and Nagy (2001) and involves cloning a PCR amplified expression cassette of interest into an intermediate shuttle vector, referred to as pLeftΔ491-2,782 or pLΔ2.4, which contains viral DNA flanking regions suitable for homologous recombination with pFAdV-9Δ4 in E. coli BJ5183 cells. The resulting plasmid would contain the FAdV-9Δ4 genome with the target expression cassette inserted into the Δ4 deletion site. When linearized and transfected into CH-SA cells, this DNA is infectious and results in virus propagation. While this method has been employed by our laboratory to generate recombinant viruses with transgenes including EGFP (Corredor and Nagy, 2010b), HA, and HN (unpublished), the overall procedure could be improved. The ability to generate a recFAdV is dependent on PCR amplification of expression cassette flanked by viral sequences, and is a subject to multiple screening procedures as the orientation of the foreign expression cassette
introduced by SwaI blunt cloning has an effect on viral transgene expression (Yang et al., unpublished). Therefore in order to avoid this problem, and in turn optimize the procedure to generate recFAdVs, I developed a new set of intermediary shuttle vectors by directionally cloning viral flanking regions into my dual-expression plasmids. The resulting intermediate constructs, termed pHMR (see Figure 3.4), contained the expression cassettes that can be easily manipulated using unique restriction enzymes (SpeI, EcoRI, NotI, and MfeI). Importantly, pHMR plasmids also contain BglII and BamHI sites that are used to extract viral-flanked expression cassettes when PCR amplification is inappropriate. PCR amplification of guanine-cytosine (GC) rich regions of DNA, for example the CAG promoter (68%), is extremely difficult and inefficient due to the formation of secondary structures. PCR reactions involving GC rich DNA often requires the addition of chemicals, such as betaine and DMSO, into the reaction buffer in order to reduce the melting temperature or the use of time-consuming protocols (Musso et al., 2006; Frey et al., 2008). Double-restriction enzyme digestion of a pHMR plasmid with both BglIII and BamHI results in a specific three band pattern after DNA gel electrophoresis. The band of interest (2,214 bp plus the size of the promoter and transgene) is gel extracted and used for homologous recombination while the remaining two bands (2082 bp and 2343 bp) are discarded. The pHMR plasmids, as new intermediate constructs provide the following benefits: 1) multiple unique RE sites for sticky-end cloning, 2) no need to screen for insert directionality as all cassettes can be designed in the rightward orientation, and 3) introduction of BamHI and BglIII sites for RE digestion and follow up fragment extraction when PCR amplification is inappropriate. Furthermore, the recombinant FAdmid DNA is subjected to NotI enzyme digestion screen as a diagnostic tool prior to transformation into DH5α cells for amplification. A distinct banding pattern is observed by agarose gel electrophoresis since four NotI sites are
present in pFAdV-9Δ4. The introduction of NotI into the pHMR expression cassettes allows for the presence of an additional diagnostic band after digestion (see Figure 3.5), and therefore makes screening for successful recombination easier. One shortfall, however, of the pHMR system is that transgenes or promoter/enhancer elements being used must not contain the unique RE sites mentioned above. This obstacle, however, can be easily overcome with site-directed mutagenesis to modify the RE site of either pHMR or the gene of interest (Carrigan et al., 2011).

Following the development of the pHMR intermediate constructs for each expression cassette, six recFAdV-9Δ4 viruses were harvested as per Corredor and Nagy (2010b). Viral growth and titer were monitored by fluorescence microscopy, and both CPE and plaque formation occurred as previously described, between 5-7 d.p.i. (Corredor and Nagy, 2010a,b). Growth kinetics of most viruses were similar to that of FAdV-9Δ4, however both the CAG and EF1α viruses had a slightly lower titer (see Figure 3.6). In recombinant adenoviruses, slower growth and a decrease in virus titer are often associated with an increased genome size relative to the wt viruses. This is most likely a product of the maximum packaging capacity of the viral capsid. In this study, FAdV-9Δ4 was the control virus as Corredor and Nagy (2010a) previously reported that its growth kinetics matched the wild-type FAdV-9 strain. In HAdV-5, recombinant viruses with genomes that are greater by over 5% (105%) of wt are unstable and replicate slowly (Bett et al., 1993). This finding is further supported in recombinant FAdV-1 (103.2%) as evident by a slower onset of CPE and a reduced titer (Francois et al., 2001). Additionally, a recombinant FAdV-9inEGFP virus (103.7%) which contains a CMV promoter driven EGFP coding region in the full length genome, also reached a half log lower titer than the wtFAdV-9 (Corredor and Nagy, 2010b). It is unlikely that this explains the reduced titer observed by both FAdV-9Δ4-CAG-EGFP (100.7%) and FAdV-9Δ4-EF1α-EGFP (100.3%), as their larger (genomic)
counterparts with slightly larger genomes, FAdV-9Δ4-CAG-EGFP-WPRE (101.9%) and FAdV-9Δ4-EF1α-EGFP-WPRE (101.5%), grew to titers as high as FAdV-9Δ4.

Promoter induced expression of EGFP by recFAdVs was assessed over 48 h.p.i. by fluorometry and Western immunoblot. Interestingly, these results using recFAdVs instead of plasmids did not support my finding in CH-SAHI cells when measuring transient expression of EGFP. Recombinant FAdVs containing CAG and EF1α expression cassettes yielded higher levels of EGFP fluorescence at each time-point compared to FAdV-9Δ4-CMV-EGFP. After two biological repeats, these findings were significant ($P < 0.05$) at all time-points at 12 h.p.i. for FAdV-9Δ4-CAG-EGFP, and extremely significantly high ($0.0001 < P < 0.001$) at 24 and 30 h.p.i. (Figure 3.8). Expression by FAdV-9Δ4-EF1α-EGFP was also significantly higher after 24 h.p.i., and extremely significant at 30 h.p.i. These findings were further supported by Western blot analysis using an anti-EGFP antibody. Based on these results, promoter strength in recFAdV infected CH-SAHI cells can be described as follows: CAG > EF1α > CMV. CH-SAHI cells infected with recFAdVs containing WPRE had lower fluorescence levels than their promoter counterparts, which was also evident when comparing EGFP band intensity after probing with an anti-GFP antibody. This result was consistent with my findings during transient expression that WPRE had a slight negative effect on transgene expression in CH-SAHI cells.

The optimization of transgene expression through testing promoters, enhancers, and regulatory elements has been studied extensively in both recombinant viruses and plasmid vectors. While these studies have focused mainly on human applications, the findings presented in this study provide a basis for improving recFAdV transgene expression. The results of my study suggest that while the CMV promoter induces high level of EGFP expression in transfected CH-SAHI cells, the expression of EGFP by recFAdVs in CH-SAHI cells can be
enhanced by using either the CAG or EF1α promoters. However, it is important to remember that promoter activity is often cell line dependent (Qin et al., 2010), and therefore further studies in other avian cell lines are warranted. The presence of WPRE in the recombinant expression cassettes negatively impacted EGFP expression in CH-SAH cells. While previous in vitro studies using recFAdV-9 vectors have demonstrated that the CMV promoter can express adequate levels of transgene (Corredor and Nagy, 2010b; Yang et al., unpublished), my findings suggest that the CAG promoter may be a more suitable option followed by the EF1α promoter, with similar findings being reported in plasmid, adenovirus, adeno-associated, and lentivirus vaccine platforms (Jiang et al., 2007; Richardson et al., 2009; Seo et al., 2010; Qin et al., 2010; Chen et al., 2011). Interestingly, a difference in CMV promoter activity in CH-SAH can be observed during transfection and infection. This decreased expression may be a result of promoter silencing by cellular methylation (Teschendorf et al., 2002, Brooks et al., 2004; Meilinger et al., 2009), which tend to be unregulated in cells during infections (Hoelzer et al., 2008).

While my findings have provided insight into improving recFAdV expression, further investigation comparing my results to that of Corredor and Nagy (2010b) is warranted as much higher levels of raw EGFP fluorescence (8.0x10^4 RFU) were previously reported for the CMV promoter. There are two major genetic differences between the CMV virus described in my study using FAdV-9Δ4-CMV-EGFP and the previous one using FAdV-9Δ4-EGFP. First, the CMV promoter used by Corredor and Nagy was PCR amplified from pEGFP-N1 (BD Biosciences Clontech) and contains only the CMV immediate early promoter/enhancer. The CMV promoter sequence in my study was PCR amplified from pCI-Neo (Promega), containing the same promoter and enhancer elements as pEGFP-N1, but a human chimeric intron was also included downstream of the promoter sequence before the EGFP coding region. Although
introns are reported to increase plasmid and viral transgene expression (Xu et al., 2001; Schiedner et al., 2002; White et al., 2008), some evidence suggest that the CMV promoter functions better without an intron (Guo et al., 1996). Secondly, the FAdV-9Δ4-EGFP virus has its expression cassette in the leftward orientation, while my virus FAdV-9Δ4-CMV-EGFP uses the rightward orientation. More recently it was determined that transgene orientation in the Δ4 deletion site greatly impacted protein expression, with cassettes in the rightward orientation measuring higher levels of protein expression compared to those in the leftward orientation (Yang et al., unpublished). While these factors should translate to improved transgene expression by a recFAdV, a comparison between both FAdV-9Δ4-EGFP (leftward orientation) and FAdV-9Δ4-CMV-EGFP (rightward orientation) in vitro could provide more insight into the differences. However, it is likely that the measured differences in raw EGFP fluorescence are the result of experimental conditions, for example cell health and passage or microplate reader used.

**Future directions**

The results described in this study provide evidence that transgene expression induced by both CAG and EF1α promoters during recFAdV-9 infection of CH-SAH cells appears to be superior to CMV promoter. Future experiments should focus on testing CMV, CAG, and EF1α promoters induced expression of EGFP in recFAdVs in other avian cell lines. Specifically, transfecting both QT-35 and DF-1 cells to measure promoter induced expression would shed light on whether the differences between my findings and Seo et al. (2010) were due to cell type. This experiment could be followed up by recFAdV infections of QT-35 and DF-1 as well. While my research has begun to optimize transgene expression in our FAdV-9Δ4 system, many additional promoters, enhancers, and regulatory elements still remain untested in avian models,
some of which may provide further improvements to recFAdVs. It would also be important to perform a transcriptional analysis by RT-PCR and Northern blot, and mRNA sequencing, for all six recFAdVs described in this study to further explain the effect of WPRE on mRNA stability and export into the cytoplasm of CH-SAH cells, as some research showed that WPRE activity is both promoter and cell line specific (Klein et al., 2006).

The potential for CAG and EF1α to modulate host immune response against EGFP should be evaluated in chickens as the application of these vectors is ultimately in birds. Previous research conducted by Corredor and Nagy (2011) studied barred rocks chickens inoculated intramuscularly with a recFAdV carrying the EGFP gene. Birds were initially inoculated with a dosage of 2 x 10^6 pfu of virus, and followed up with homologous boosts at 2, 3, and 4 weeks post initial inoculation. Antibodies to EGFP were found in recFAdV groups at 3-7 weeks, suggesting that boosting was important when eliciting an immune response to EGFP. This finding correlated with previous studies, although not in chickens, where EGFP has been shown to be poorly immunogenic (Skelton et al., 2001). Additionally, Corredor and Nagy found plausible evidence that the genetic background of chickens influenced the immune response to recFAdVs, similarly to studies conducted in white leghorns (Ojkić and Nagy, 2003). More recently, Deng et al. (2013) studied the immune response of orally inoculated SPF white leghorn chickens with the FAdV-9Δ4. Unlike intramuscular inoculation, oral inoculation simulates the natural fecal-oral route of infection. Although previous oral administration of FAdV-9 has been reported by Ojkić and Nagy (2003), in this study virus was provided in both the water and feed and therefore exact dosage to each bird could not be accounted for. Deng et al. (2013) found that after a single oral inoculation of 1.5 x 10^7 pfu, the results were similar with previous intramuscular inoculation trials comparing FAdV-9Δ4 and wt FAdV-9, where FAdV-9Δ4 replicated less efficiently in vivo
than the wt as evidenced by reduced virus shedding in the feces, lower genome copy number in tissues, and a lower IgG antibody response. Intramuscular vaccination is not a preferred route for the poultry industry, therefore animal trials should focus on oral or in ovo methods of delivery. Optimization of both delivery and administration of AdV-based vaccines are crucial for both humans and animals (Thacker et al., 2009).

To study how all six of my recFAdV viruses stimulate the host immune response to EGFP, I would propose a study to orally inoculate SPF chickens to measure IgG to EGFP by ELISA (Carter and Kerr, 2003; Corredor and Nagy, 2011). Further, I would continue to collect blood past the 7 weeks post-inoculation previously reported, to determine the week post-inoculation at which IgG against FAdV-9Δ4 begins to decrease (see preliminary data in Appendix 2 and 3). Data of my in vitro experiments suggest that birds inoculated with viruses containing the WPRE element would have a decreased level of IgG to EGFP when compared to the promoter only groups. Although, Li et al. (2013) inoculated SPF chickens with DNA vaccines towards infectious bursal disease, which included the CAG promoter expressing the VP243 gene and a WPRE element, they reported that the presence of WPRE significantly increased antigen expression, immune response, and conferred protection to IBDV. Other research groups showed that WPRE can improve humoral immunity 2-fold in BALB/c mice inoculated intramuscularly with DNA vaccines containing influenza virus HA (Garg et al., 2004) or baculovirus-mediated HA (Chen et al., 2013). Nevertheless, it is important to note that the effect of WPRE in virus vectors has not yet been measured in poultry, and therefore in the context of FAdV infection, WPRE may still have an adverse effect on EGFP expression as demonstrated in CH-SAH cells. The secreted mucosal IgA levels could be measured by collecting intestine and lung tissue samples and performing indirect ELISA, such as that
described by Chaudhari et al. (2013).

Future research should further investigate the molecular biology of FAdVs as we still do not know the function of all the early ORFs. Deng et al. (2013) provided evidence that the ORFs deleted from FAdV-9Δ4 could explain the variance in cytokine regulation compared to wt FAdV-9. If we understood the function of each ORF deleted in our virus backbone, we could determine if re-introducing certain ORFs improve the function of our vaccine platform.

Conclusions

This study investigated a variety of promoters and one regulatory element to improve foreign gene expression from recFAdV vectors. First, the promoter activity in transfected CH-SAH cells was compared by dual-expression assay and it was found that the CMV promoter induced the highest level of expression and that WPRE acted negatively on expression. Further analysis of the CMV, CAG, and EF1α promoters occurred in CH-SAH cells infected with recombinant viruses that were generated with the help of a new more efficient intermediate construct system, which I developed. EGFP expression in CH-SAH cells was measured by fluorometry and Western immunoblot. Both the CAG and EF1α promoters expressed much higher levels of EGFP than the CMV promoter, and viruses containing WPRE expressed EGFP at slightly lower levels than their promoter only counterparts. These results suggest that recFAdV expression in CH-SAH cells can be improved from the current system by using either the CAG or EF1α promoters, which is consistent with current literature in mammalian systems.

Control of avian disease through vaccination remains a hallmark strategy for protecting poultry against current and future threats. FAdV based vaccines offer promise for enhancing poultry health. Understanding how to optimize and enhance protein expression could lead to
more efficient vaccines, including those against highly pathogenic AI and NDV. The results described in this thesis provide a novel insight into the field of FAdV vector design and transgene specific optimization.
References


Washietl, S., Eisenhaber, F. 2003. Reannotation of the CELO genome characterizes a set of previously unassigned open reading frames and points to novel modes of host interaction in avian adenoviruses. BMC Bioinformatics. 4:55.


## Appendices

### Appendix 1.

Fold change in promoter activity of transfected CH-SA2 cells at various hours post-transfection in relation to the CMV promoter. Significant activity is determined by a \( P \) value <0.05.

<table>
<thead>
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<th>SD</th>
<th>( P ) value</th>
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SD = Standard deviation  
Δfold = Average fold change in activity
### Appendix 2. Viral shedding (pfu/ml) in the feces of chickens inoculated with recFAdV-9Δ4s

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<th>d.p.i</th>
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<th>FAdV-9Δ4- CMV-EGFP-WPRE$^A$</th>
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$^A$20 birds tested  
$^B$10 birds tested  
$^C$ND, not detected
Appendix 3. IgG antibody response to FAdV-9 proteins in chickens inoculated with $5.0 \times 10^6$ pfu of recFAdVs (coloured), FAdV-9Δ4 (white bars), and PBS mock-infected chickens (black bars) as measured by sample-to-positive reactions. The error bars correspond to 95% confidence intervals. The arrow indicates a boost inoculation.