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Fowl adenovirus 9 ORF19, a lipase homolog, is nonessential for virus replication and is suitable for foreign gene expression

Yanlong Pei[a], Juan C. Corredor[a], Peter J. Krell[b], Éva Nagy[a,*]

[a] Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Canada
[b] Department of Molecular and Cellular Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario, N1G 2W1, Canada

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ABSTRACT

Fowl adenovirus 9 (FAdV-9) has one of the largest genomes (45 kb) so far sequenced from all adenoviruses studied. Genus-specific genes located within the early (E) regions at the right and left ends of the viral genome have unknown functions except for ORF8 (*Gam-1* gene), ORF22 and ORF1 (*dUTPase* gene). ORF19, located at the right end of the genome (nts 34,220-36,443), is predicted to encode a lipase protein and its homologs are also found in all FAdV genomes so far sequenced. The role of ORF19 in virus replication and virulence is unknown. To study ORF19 and explore its potential as a locus for foreign gene insertion, we generated one ORF19-deleted mutant virus (rFAdV-9Δ19-SwaI) and three FAdV-9Δ19-based recombinant viruses replacing ORF19 as follows: rFAdV-9Δ19-CAT and enhanced-green fluorescent protein (EGFP) cassette (CMV promoter-EGFP-poly A) in a rightward (rFAdV-9Δ19-EGFP-R) and leftward orientation (rFAdV-9Δ19-EGFP-L). All recombinant viruses were stable after three passages. In chicken hepatoma cells, rFAdV-9Δ19-SwaI, rFAdV-9Δ19-CAT and rFAdV-9Δ19-EGFP-R replicated at titers similar to that of the wild-type virus, whilst rFAdV-9Δ19-EGFP-L replicated at a much lower titer. Interestingly, FAdV-9Δ19-SwaI replicated at higher titers in cells and in embryonated eggs, respectively than those of wild-type and recombinant viruses. These observations suggest ORF19 is nonessential for replication and can be used as a novel cloning site for engineering FAdV-9-based recombinant viruses and rFAdV-9Δ19-SwaI could be used to determine its role for virus replication *in vivo*.

1. Introduction

Fowl adenovirus 9 (FAdV-9) belongs to species *Fowl aviadenovirus D* in the genus *Aviadenovirus*. In general, aviadenoviruses have the largest DNA genomes among members of the adenovirus genera (around 10 kb larger than that of mastadenoviruses). The majority of genus-specific genes at the left (nts 1–6,128) and right ends (nts 31,877-45,063) of the viral genome are conserved among aviadenoviruses, with some variations in gene content (Davison et al., 2003; Corredor et al., 2006, 2008; Marek et al., 2014a, 2014b, 2016). The function of early genes located at the left and right end regions is unknown, except for ORF1 (*dUTPase*), ORF8 (*Gam-1*) and ORF22 (Chiocca et al., 1997; Deng et al., 2016; Lehrmann and Cotten, 1999). Nineteen ORFs and two tandem repeat regions (TR-1 and TR-2) map to the left and right ends of the FAdV-9 genome (Ojkic and Nagy, 2000). One of the right end ORFs, ORF19 (nts 34,220-36,443), encodes a polypeptide with predicted lipase function (Ojkic and Nagy, 2000). ORF19 homologs are present as a single copy with leftward orientation in most aviadenoviruses (Corredor et al., 2008; Marek et al., 2014a, 2014b, 2016). However, variations in the number, genomic position and orientation for ORF19 were found in the genomes of several aviadenoviruses including duck adenovirus 2 and goose adenovirus 4 in which ORF19 is duplicated, both in a leftward orientation. In the genomes of FAdV-4, FAdV-10 and turkey adenovirus 4 and 5, ORF19 is also duplicated but in opposite orientations (Corredor et al., 2008; Griffin and Nagy, 2011; Kaján et al., 2012; Li et al., 2016; Marek et al., 2014b).

Viral lipase (vLIP) is a virulence factor for the Marek's disease herpesvirus, albeit its expression is not required for *in vitro* replication (Kamil et al., 2005). The lipase activity of certain other viral proteins, such as parvovirus capsid proteins VP1 have been found to be essential for virus replication (Zádori et al., 2001). Recent genome sequence analyses and comparisons of FAdV-4 isolates from clinical cases of inclusion body hepatitis and hydropericardium syndrome suggested that abrogation and/or alterations of ORF19 expression are associated with virulence (Liu et al., 2016; Pan et al., 2017; Vera-Hernández et al., 2016; Ye et al., 2016; Zhao et al., 2015). However there is no experimental evidence showing that abrogation of ORF19 expression directly affects virulence or pathogenesis.

* Corresponding author.
E-mail address: enagy@uoguelph.ca (É. Nagy).

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FAdV-9 (strain A-2 A) is non-pathogenic (Ojkic and Nagy, 2003) and its ORF19 homolog is transcribed early in infection (Ojkic et al., 2002). To determine whether ORF19 is essential for virus replication and if abrogation of its expression would affect virulence, we generated one ORF19-deleted mutant (FAdV-9Δ19-SwaI) from which three FAdV-9Δ19-based recombinant viruses were generated. We found that ORF19 is dispensable for virus replication *in vitro* in chicken hepatoma (CH-SAH) cells and *in vivo* (in embryonated eggs). Foreign, enhanced green fluorescent protein (EGFP), gene expression was detected in CH-SAH cells infected with the recombinant viruses. Interestingly, the ORF19-deleted virus, FAdV-9Δ19-SwaI, grew to higher titers than the wild-type (wt) virus, both in CH-SAH cells and in allantoic fluids of chicken embryos. Chicken embryos did not show any gross pathological signs when infected with any of these viruses. These observations collectively suggest that deletion of the FAdV-9 lipase homolog, ORF19, slightly increases virulence, but does not influence pathology and that ORF19 is not essential for virus replication. In addition, we show that the ORF19 locus is a site suitable for foreign gene cloning and generation of recombinant viruses, potentially as vaccines.

2. Materials and methods

2.1. Cells and viruses

FAdV-9, strain A-2 A (ATCC VR-833), and all derivatives were propagated in CH-SAH cells as described (Alexander et al., 1998). Supernatant from the infected cells was used as the source of extracellular virus for all experiments. Virus titers were determined by plaque assay and all viruses were used at a multiplicity of infection (MOI) of 5 for cell infections.

2.2. Generation of ORF19-deleted mutant and recombinant viruses

The FAdmid backbone (infectious clone consisting of the viral genome cloned into a cosmid vector) was used for chloramphenicol acetyl transferase (CAT) gene-mediated targeted deletion of ORF19 (pFAdV-9Δ19-SwaI) using procedures as described (Corredor et al., 2017; Pei et al., 2015). Briefly, the CAT cassette was PCR amplified with forward primer ORF19CAT-SwaI-F and reverse primer ORF19CAT-SwaI-R (Table 1). Both primers contained a 50–52 nucleotide arm of the ORF19 upstream and downstream sequences, SwaI sites and the first 20–23 nucleotides for the CAT cassette. Competent *Eschericia coli* DH10B cells were co-transformed with both gel purified CAT amplicon and FAdmid9. Bacteria were grown on LB plates containing both chloramphenicol and ampicillin. The CAT cassette was subsequently removed by SwaI digestion followed by religation to generate construct pFAdV-9Δ19-SwaI. To generate recombinant FAdmids pFAdV-9Δ19-EGFP-R and pFAdV-9Δ19-EGFP-L, pFAdV-9Δ19-SwaI was digested with SwaI and the EGFP cassette (CMV-EGFP-polyA signal) was cloned in rightward (R) and leftward (L) orientations, respectively. The resulting FAdmid constructs were verified by NotI digestion and PCR amplification. Two μg of each FAdmid was digested with PacI to linearize the DNA, and transfected into CH-SAH cells with Lipofectamine (Life Technologies). ORF19-deleted mutant virus (rFAdV-9Δ19-SwaI) and recombinant viruses (rFAdV-9Δ19-CAT, rFAdV-9Δ19-EGFP-R and rFAdV-9Δ19-EGFP-L) were rescued at 7 days post-transfection.

2.3. Virus passage and one-step growth curves

To determine the stability of the viruses, they were passaged through three cycles and viability tested by plaque assay. Passage 0 was virus from the initial FAdmid-transfected cells collected at 7 days post-transfection. For each subsequent passage, CH-SAH cells in 25 cm^2 flasks were inoculated with 200 μl of supernatant from virus-infected cells collected at 5 days post-infection (pi) from the previous passage. For virus growth curves, CH-SAH cells were infected with rFAdV-9Δ19-CAT, rFAdV-9Δ19-SwaI, rFAdV-9Δ19-EGFP-R, rFAdV-9Δ19-EGFP-L and wtFAdV-9 at an MOI of 5. Samples were collected every 6 h (from 0 to 54 h) with time 0 representing one hour after virus was first added to the cells. For each time point, cells were centrifuged at 6000 *g* for 15 min. at 4 °C and virus was recovered from the supernatants. Virus titrations were done in duplicate as reported previously (Alexander et al., 1998). Virus titers were expressed as plaque forming units (PFU)/ml. Viral genome copy numbers in virus supernatants were determined by PCR of a 114 bp region including ORF20 A as described (Romanova et al., 2009).

2.4. Virus replication in embryonated eggs

We have previously shown that the replication of deleted-mutant viruses in chickens correlates with replication ability in embryonated eggs demonstrating that embryonated eggs can be used as an experimental alternative to chickens (Corredor and Nagy, 2010a). To test the replication of the different virus constructs in chicken embryos, 128, 10-day-old embryonated chicken eggs were divided into five groups of 24 each for virus infection (rFAdV-9Δ19-SwaI, rFAdV-9Δ19-CAT, rFAdV-9Δ19-EGFP-R, rFAdV-9Δ19-EGFP-L and wtFAdV-9) and one uninfected control group of 8. Eggs were inoculated with 100 μl virus (1×10^5 PFU) *via* the allantoic route. Eggs in the negative control group were inoculated with 100 μl PBS. Allantoic fluid from 6 eggs of each infection group and two eggs from the negative control group were harvested at each of four time points, 24, 48, 96 and 120 h post-inoculation (hpi) and titered in duplicate in CH-SAH cells as reported (Alexander et al., 1998). Statistical significance was determined using a 2-tailed Student's t-test.

Table 1
The primers used in this study.

Name	Sequence (5'-3')	Location	Purpose
ORF19CAT-SwaI-F	ccgcccgtcacaaccgcgatacgcgtaactctatcttccc	34168-34219	CAT cassette amplification replacement of ORF19, introduction of SwaI sites
ORF19CAT-SwaI-R	aaaattcacggaATTTAAAT**gtgtaggctggagctgcttc** gctgtctcagattcaggtggactgttgtgcggctcttgcc ccagatcttcATTTAAAT**catatgaatatcctccttagttc**	36444-36493	
Ver-ORF19-F	caactgactacggaatacaggg	33698-33719	verification
Ver-ORF19-R	ctagtttgcttccgcgtacg	36846-36886	
EGFPcaSwaI-F	*agctgc*ATTTAAAT<u>gtattaccgccatgcattag</u>	4717-3	EGFP cassette amplification
EGFPcaSwaI-R	*agctgc*ATTTAAAT<u>ccacaactagaatgcagtg</u>	1597-1615	

Restriction enzyme sites are capitalized; Sequences in bold are chloramphenicol cassette specific; Underlined sequences are pN1-EGFP specific, the location is based on pEGFP-N1; The location of FAdV-9 specific primers is based on GenBank No. AC_000013; Italicized sequences are extra nucleotides for restriction enzyme digestion.

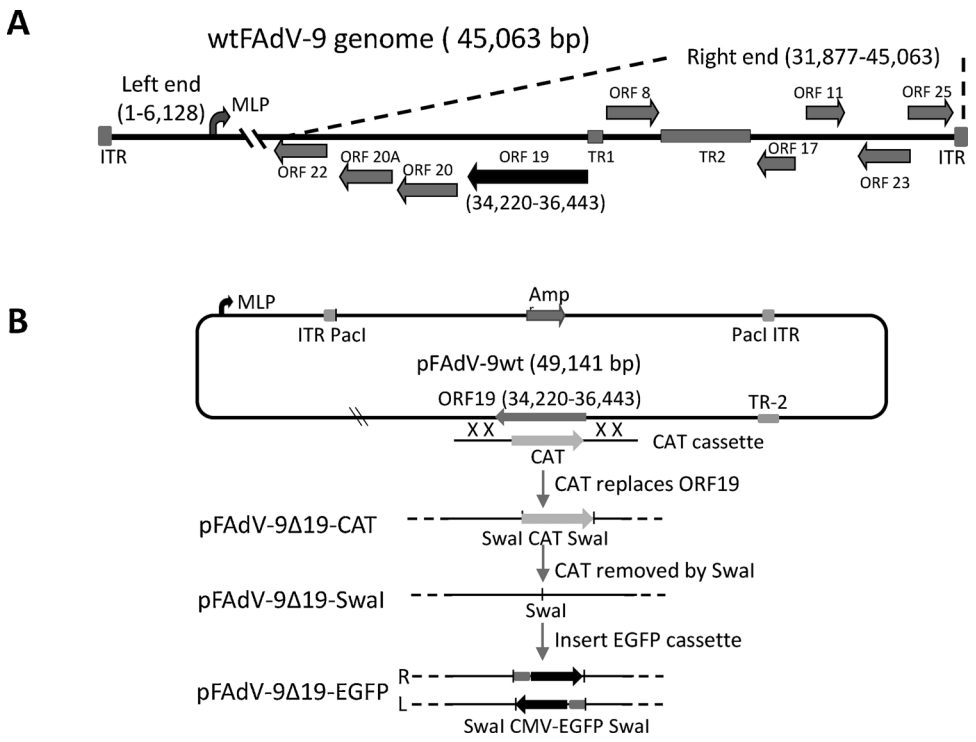

Fig. 1. Genome map, expanded at the right end (A) and flowchart (B) for the generation of four pFAdV-9 ORF19-deleted FAdmids. The entire ORF19 sequence (nts 34,220-36,443) was replaced with the CAT cassette, flanked on both sides with SwaI sites, by homologous recombination to generate pFAdV-9Δ19-CAT. The CAT cassette was removed by SwaI digestion followed by religation to generate unmarked pFAdV-9Δ19-SwaI. An EGFP cassette (CMV promoter-EGFP-polyA) was cloned into pFAdV-9Δ19-SwaI to generate pFAdV-9Δ19-EGFP-R and pFAdV-9Δ19-EGFP-L. ITR: inverted terminal repeat; MLP: major late promoter.

3. Results

3.1. Generation of four ORF19-deleted FAdmids

The flowchart for the generation of the four pFAdV-9 ORF19-deleted FAdmids is presented in Fig. 1. FAdmid DNA constructs were verified by NotI digestion and PCR amplification with verification primers ver-ORF19-F and ver-ORF19-R (Table 1, Fig. 2). The sizes of DNA fragments for NotI digestions (Fig. 2A) and PCR amplification products (Fig. 2B and C) were as expected, equivalent to those given in the legend to Fig. 2.

3.2. Virus rescue and replication

Cytopathic effects (CPE) in CH-SAH cells were evident at 5–7 days post-transfection with 2 μg PacI linearized FAdmids for each of the four constructs. The infectivity of all four rescued viruses was confirmed up to three passages. The titers of passage three rFAdV-9Δ19-CAT, rFAdV-9Δ19-SwaI, rFAdV-9Δ19-EGFP-R, rFAdV-9Δ19-EGFP-L at 5 days pi were 1.5×10^8, 5.5×10^8, 8.5×10^7 and 3.5×10^6 PFU/ml, respectively while the titer of the wt virus was 2.5×10^8 PFU/ml. The equivalent genome copy numbers for rFAdV-9Δ19-CAT, rFAdV-9Δ19-SwaI, rFAdV-9Δ19-EGFP-R, rFAdV-9Δ19-EGFP-L and wtFAdV-9 were also determined and were 6.70×10^{10}, 6.25×10^{10}, 4.41×10^{10}, 2.38×10^{10} and 3.93×10^{10} viral genomes/ml. As indicated by the one-step growth curves, rFAdV-9Δ19-SwaI at 54 hpi yielded the highest titer, which was significantly different from the other viruses including the wt virus (Fig. 3). There were no significant differences among the titers obtained for wtFAdV-9, rFAdV-9Δ19-CAT and rFAdV-9Δ19-EGFP-R. However, rFAdV-9Δ19-EGFP-L replicated to a lower titer compared to wtFAdV-9, rFAdV-9Δ19-SwaI, rFAdV-9Δ19-CAT and rFAdV-9Δ19-EGFP-R and was statistically different from them. The overall replication kinetics were the same for all viruses. At days 5–7 pi, no differences could be observed by light microscopy in the CPE and plaque morphologies generated by any of the examined viruses.

Although ORF19 is considered a lipase homolog, it is not known if it is functional as a lipase. To address this question, lipase activity in infected and uninfected cells was compared reasoning that if the FAdV ORF19 is functional as a lipase then higher levels of activity in cell extracts would be found in wt virus infected cells and lower but

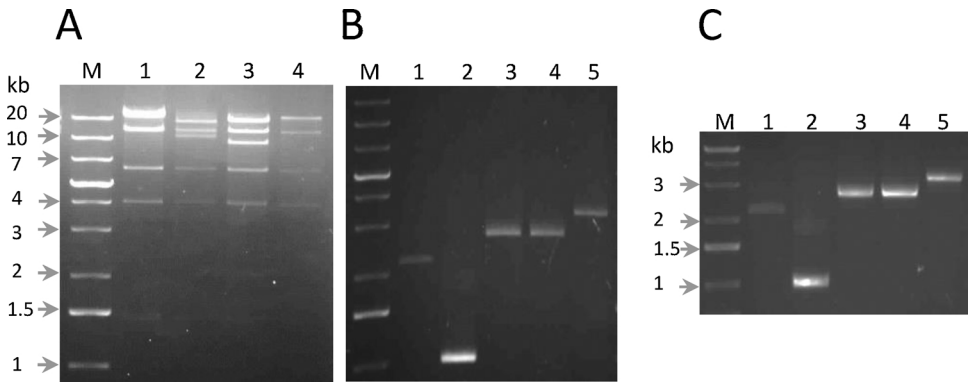

Fig. 2. Verification of FAdmid constructs by NotI digestion (A) and PCR amplification (B) and verification of stability of passage three virus genomes by PCR amplifications (C). Lanes M: 1 kb DNA ladder. Panel A: lane 1 pFAdV-9Δ19-SwaI (expected fragment sizes are 25,058 bp, 11,485 bp, 6056 bp, 4078 bp and 1391 bp); lane 2 pFAdV-9Δ19-EGFP-R (expected fragment sizes are 15,517 bp, 11,485 bp, 10,022 bp, 6056 bp, 4078 bp and 1391 bp); lane 3 pFAdV-9Δ19-EGFP-L (expected fragment sizes are 16,689 bp, 11,485 bp, 8850 bp, 6056 bp, 4078 bp and 1391 bp) and lane 4 pFAdV-9 wt (expected fragment sizes are 11,485 bp, 10,022 bp, 6056 bp, 4078 bp and 1391 bp). Panels B and C respectively PCR products: lane 1 pFAdV-9Δ19-CAT or passage three viruses (2115 bp); lane 2 pFAdV-9Δ19-SwaI or passage three virus (972 bp); lane 3 pFAdV-9Δ19-EGFP-R or passage three virus (2597 bp); lane 4 pFAdV-9Δ19-EGFP-L or passage 3 virus (2597 bp), and lane 5 pFAdV-9 wt or passage three virus (3189 bp).

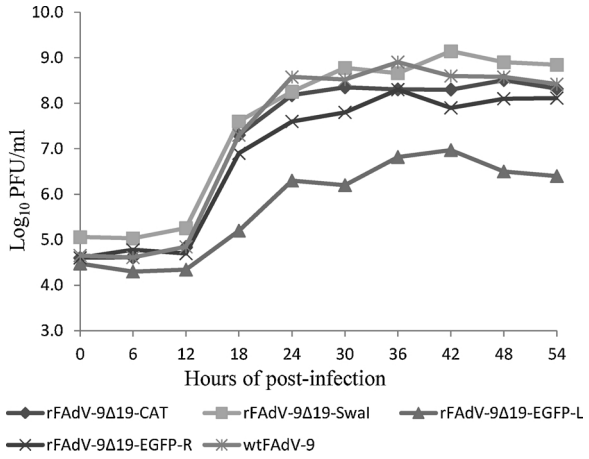

Fig. 3. One-step growth curves. Chicken hepatoma cells (CH-SAH) were infected with wild-type (wt), rFAdV-9Δ19-CAT, rFAdV-9Δ19-SwaI, rFAdV-9Δ19-EGFP-R and rFAdV-9Δ19-EGFP-L viruses at an MOI of 5, and infected cell supernatants were harvested at the indicated time points and titered by plaque assay. Virus titers were determined in two technical repeats and expressed as PFU/ml. Time 0 is taken as 1 h after addition of virus to the cells.

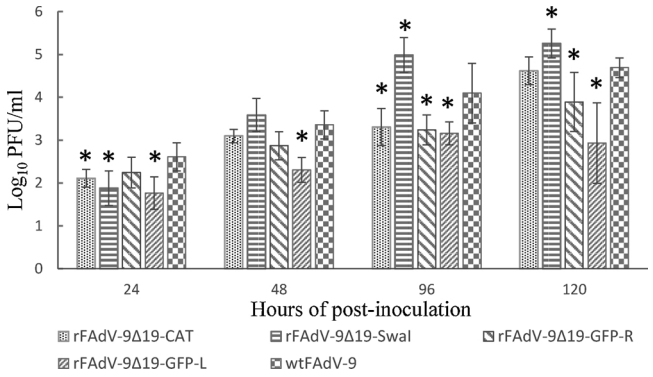

Fig. 5. Replication of recombinant viruses and wild-type virus in 10-day-old chicken embryonated eggs. Allantoic fluids were collected at 24, 48, 96 and 120 h post-inoculation from six embryonated eggs each inoculated with 1×10^5 PFU of rFAdV-9Δ19-CAT, rFAdV-9Δ19-SwaI, rFAdV-9Δ19-EGFP-R, rFAdV-9Δ19-EGFP-L and wtFAdV-9, respectively. Virus titers (PFU/ml) were determined in duplicate by the plaque assay. Data are presented as means and ± standard deviations. Asterisks indicate values, which were significantly different from the wild-type virus.

equivalent levels found in uninfected cells or in cells infected with an ORF19 deleted virus. Using either CH-SAH cells (in which FAdV replicates) or DF-1 cells (in which FAdV does not replicate) we found no significant differences in lipase activities in the supernatants of either wt or rFAdV-9Δ19 virus infected cells or uninfected control cells at either 24 or 48 hpi. Lipase activities were somewhat higher in cell extracts of wt and rFAdV-9Δ19-SwaI virus infected cells or uninfected control cells at 24 hpi with lysates from wt virus-infected cells being the lowest. By 48 hpi levels of lipase activity in cell lysates from both virus-infected cells dropped and were not significantly different from each other (Supplemental Fig. 1).

3.3. Foreign gene expression

EGFP expression was detected by fluorescence microscopy in CH-SAH cells infected with rFAdV-9Δ19-EGFP-R and rFAdV-9Δ19-EGFP-L (Fig. 4). The stability of the foreign gene in the recombinant viruses was monitored by EGFP expression up to the third viral passage. EGFP signals with similar intensity were observed for all 15 randomly selected plaques for each virus, as evaluated by visual assessment.

3.4. Virus replication in embryonated eggs

Allantoic fluids from 10-day-old embryonated eggs infected with the different viruses were harvested at 24, 48, 96 and 120 hpi and titrated in CH-SAH cells (Fig. 5). No virus was detected in the PBS-inoculated control group. By 120 hpi the mean titers of the allantoic fluids for rFAdV-9Δ19-CAT, rFAdV-9Δ19-SwaI, rFAdV-9Δ19-EGFP-R, rFAdV-9Δ19-EGFP-L and wtFAdV-9 were 4.1×10^4 PFU/ml, 1.8×10^5 PFU/ml, 7.8×10^3 PFU/ml, 8.6×10^2 PFU/ml and 4.9×10^4 PFU/ml, respectively. rFAdV-9Δ19-SwaI replicated at higher titers with respect to even the wt FAdV-9 at 96 and 120 hpi and these differences were significant ($P = 0.007$ and $P = 0.005$, respectively). By 120 hpi the titer of the ORF19-deleted virus (rFAdV-9Δ19-SwaI) was 3.7 fold higher than the wt virus, reflecting a similar fold higher level in rFAdV-9Δ19-SwaI-infected CH-SAH cells. The titer of rFAdV-9Δ19-CAT was not significantly different from wt virus throughout the course of this study, whilst recombinant virus rFAdV-9Δ19-EGFP-R, replicated at lower titers that were significantly different from each other ($P = 0.012$) at 48 and 120 hpi. rFAdV-9Δ19-EGFP-L replicated at the lowest level compared to all others at values that were significantly different from rFAdV-9Δ19-SwaI and wtFAdV-9 at 120 hpi ($P = 0.006$ and $P = 0.008$, respectively).

4. Discussion

The fowl adenovirus genome, and likely genomes of most aviadenoviruses, contains dispensable regions that are amenable for manipulation and generation of recombinant viruses in cell culture. We have identified such regions at the left and right ends of the FAdV-9 genome (nts 481-2,782 and nts 38,307-42,398, respectively). Those regions include ORFs 0, 1, 1 A, 1B, 1C and 2 at the left end region and ORFs 17 and 11 and TR-2 at the right end region (Corredor and Nagy, 2010a, b;

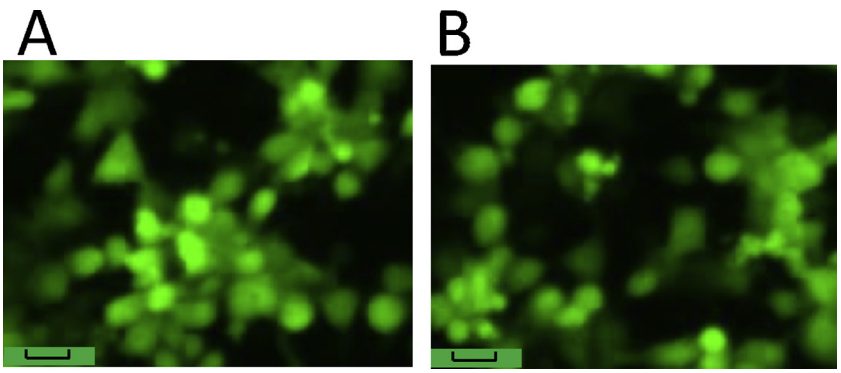

Fig. 4. EGFP expression by fluorescence microscopy in CH-SAH cells infected with rFAdV-9Δ19-EGFP-R (A) and rFAdV-9Δ19-EGFP-L (B). Fluorescence images were taken at day 3 post-infection. The scale bars in the lower left corner of the images represent 10 μm.

Ojkic and Nagy, 2001; Pei et al., 2018a). Recently, we have demonstrated that ORFs 16 and 17 of FAdV-4 are dispensable for virus replication and suitable for the generation of recombinant viruses, though the ORF16-17-deleted mutant virus replicates to a lower titer (Pei et al., 2018b). This might make it a less suitable vector for gene expression and vaccine development, although we showed recently (Ackford et al., 2017) that efficacy of a FAdV-9 vector based vaccine did not depend on the level of foreign gene expression.

ORF19 encodes a polypeptide with predicted lipase function and its homologs are present in all aviadenovirus genomes sequenced so far (Corredor et al., 2008; Griffin and Nagy, 2011; Li et al., 2016; Marek et al., 2014a, 2014b, 2016; Ojkic and Nagy, 2000). The amino acid sequence of ORF19 of FAdV-9 is most similar to the counterparts in FAdV types and isolates also belonging to species FAdV-D (94–96% identities). The similarities to members of species FAdV-E are somewhat lower with 59–62% identities but even lower with viruses in the other FAdV species A, B and C with identities of only 27–32%. Nonetheless the putative lipase domain is preserved in every virus (Corredor et al., 2008; Grgić et al., (2011); Slaine et al., 2016; Chiocca et al., 1996; Marek et al., 2013; Zhao et al., 2015). That the lipase activity in wt virus infected cells was not greater than either mock infected cells or cells infected with rFAdV-9Δ19 suggests ORF19 does not encode a functional lipase, as demonstrated for a Marek's disease virus lipase homolog (Kamil et al., 2005), or that if there was any viral lipase activity it was below the level of detection.

Since the association of virus virulence and alterations of ORF19 expression have not been demonstrated experimentally, we formulated a hypothesis that deletion of ORF19 in the genome of non-pathogenic FAdV-9 would result in increased virulence. Interestingly, rFAdV-9Δ19-SwaI, lacking ORF19 replicated at higher levels, up to 2.2 fold *in vitro* and up to 3.7 fold *in vivo* than even wt virus in embryonated eggs (96 and 120 hpi) but with no pathological signs in the embryos. These observations suggest that abrogation of ORF19 expression increases the virulence for FAdV-9 both *in vitro* and *in vivo*, but has no effect on pathogenesis. Unlike the lipase homolog in Marek's disease virus which appears to be a virulence factor, though not functional as a lipase (Kamil et al., 2005), the lipase homolog in FAdV-9 does not appear to be a virulence gene and to the contrary, it is the deletion of this gene which increases virulence. Thus the molecular basis of virulence in aviadenoviruses remains unclear. The fiber protein of FAdV-8 was the first protein suggested as a virulence factor (Pallister et al., 1996). However, sequence comparison of fiber genes of pathogenic and non-pathogenic FAdV-8 and FAdV-11 isolates did not show any differences between them (Grgić et al., 2014; Slaine et al., 2016). Deletions in the right end region including a tandem repeat (TR-E) and some ORFs (ORFs 19, 27, 29) are thought to be associated with FAdV-4 virulence (Liu et al., 2016; Pan et al., 2017; Vera-Hernández et al., 2016; Zhao et al., 2015). FAdV-4 has two ORF19 homologs in opposite orientations (ORF19 and ORF19 A, respectively), whilst FAdV-9 has only one ORF19 homolog, which is in a leftward orientation (Corredor et al., 2008). So far, no mutations that result in either alteration or abrogation of ORF19 A expression have been reported in pathogenic species D isolates.

The function of ORF19 in virus replication remains unknown, though interestingly its deletion enhances virulence, up to 3.7 fold *in vivo* and 2.2 fold *in vitro*. Replacement of ORF19 with the EGFP cassette in the L orientation, on the other hand, dramatically decreased virus yield, up to 57 fold *in vivo* and up to 200 fold *in vitro*. Although virus yield varied somewhat for the different viruses, there were no differences in the level of viral genome equivalents, suggesting that ORF19 has no role in viral DNA synthesis. Replacing ORF19 with EGFP in the R orientation also decreased virus yield, though less dramatically, about 6 fold *in vivo* and about 3 fold *in vitro* compared to wt virus. In our previous study replacement of right end ORFs 16 and 17 in FAdV-4 with the EGFP cassette in the leftward orientation also caused a major reduction in virus replication relative to virus with the EGFP in the rightward orientation (Pei et al., 2018b). The leftward transcription from the exogenous CMV promoter (in the EGFP cassette) may have reduced virus replication due to an anti-sense effect on late transcription from the major late promoter for right oriented viral genes for structural proteins. All these observations suggest that better transgene expression can be achieved when the foreign gene expression cassette is cloned in the rightward orientation.

In conclusion, our data demonstrated that ORF19 of FAdV-9 is dispensable for virus replication and the site is suitable for foreign gene insertion and expression. In addition, deletion of ORF19 enhanced virus replication in both CH-SAH cells and embryonated chicken eggs but had no effect on pathogenesis. Our work suggests that in addition to ORF19, alterations of viral gene expression as a whole, either by nucleotide substitutions or deletion, could result in a gain of virulence. Our ORF19 deletion also provides a useful reagent to determine the role of the lipase homolog during infection and perhaps provide a clue about why its deletion enhances virus replication.

Conflict of interest

All authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2018.12.001.

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