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Selection of differently temporally regulated African swine fever virus promoters with variable expression activities and their application for transient and recombinant virus mediated gene expression

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ABSTRACT

African swine fever virus threatens pig production worldwide due to the lack of vaccines, for which generation of both deletion and insertion mutants is considered. For development of the latter, operational ASFV promoters of different temporal regulation and strengths are desirable. We therefore compared the capacities of putative promoter sequences from p72, CD2v, p30, viral DNA polymerase and U104L genes to mediate expression of luciferase from transfected plasmids after activation *in trans,* or p30-, DNA polymerase- and U104L promoters *in cis*, using respective ASFV recombinants. We identified sequences with promoter activities upstream the viral ORFs, and showed that they differ in both their expression intensity regulating properties and in their temporal regulation. In summary, p30 and DNA polymerase promoters are recommended for high level early regulated transgene expression. For late expression, the p72, CD2v and U104L promoter are suitable. The latter however, only if low level transgene expression is aimed.

1. Introduction

African swine fever virus (ASFV) causes ASF, a contagious and lethal disease of domestic swine and wild boar that constitutes a substantial threat for the pig husbandry worldwide. It is the only known arbovirus with a DNA genome and the only member of the family *Asfarviridae*, genus *Asfivirus*. ASF, listed as OIE-notifiable disease, is of high socio-economic impact for affected countries because of high losses in pig production and trade. The first description of ASF dates back to 1921 (Montgomery, 1921), with the disease being introduced from Africa into Europe in 1957 and 1960. With the exception of Sardinia where it became endemic, ASF was eradicated from Europe in the ensuing decades. In 2007 it was introduced into the Caucasus region, from where it continuously spread into the neighbouring countries until in 2014 it reached Poland, Lithuania, Latvia and Estonia (Guinat et al., 2016). ASF still lacks a vaccine for prevention, which restricts control to culling and quarantine measures.

ASFV is a large and complex virus with a double stranded DNA genome of 170–190 kbp in size and, depending on the strain, contains150 to 167 ORFs. Encoded proteins are involved not only in viral replication and morphogenesis but also in modulation of host cell functions and immune evasion (Correia et al., 2013; Dixon et al., 2004, 2013). ASFV replicates in specialized cytoplasm structures, the viral factories, which develop in the vicinity of the host cell nucleus from approximately 4 h after infection (Brookes et al., 1996; Heath et al., 2001; Nunes et al., 1975). The viral replication cycle is split into an early phase, prior to viral DNA replication, and a late phase that begins after its onset at approximately 6 h to 8 h post-infection (p.i). Most of the viral gene expression, DNA replication and morphogenesis take place in the viral factories, except for early synthesis of viral DNA precursors that has been observed to occur in the nucleus of the host cells (Rojo et al., 1999; Simoes et al., 2015). The viral genome codes for the necessary enzymes for viral gene transcription and DNA replication (reviewed in Dixon et al., 2013). Four classes of expression have been described for ASFV genes. Viral DNA replication defines the transition from immediate early and early to intermediate and late gene transcription, as assessed by analysis of mRNA collected at different time-points of infection and in the presence of inhibitors of DNA or protein synthesis (reviewed in Rodriguez and Salas, 2013; Salas et al., 1986). Immediate early genes are repressed before the onset of DNA replication, in a process that requires protein synthesis, while early genes continue to be transcribed until replication takes place, declining soon after. Transcription of intermediate genes starts immediately after DNA replication and gradually declines afterwards, and for late genes it starts after the intermediate ones, reaching maximal levels at 12 h to 16 h p.i. and decreasing slowly until the end of the infection cycle,

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Table 1
Oligonucleotide pairs used for construction of transient luciferase expression plasmids. Areas corresponding to viral sequences are in bold; restriction enzyme sites used in the cloning process are underlined. The viral sequence strain used as reference for oligonucleotide pair nº3 was strain NHV (Portugal et al., 2015. Genbank accession: KM262845.1); for the remaining pairs it was ASFV-L60 (Portugal et al., 2015. Genbank accession: KM262845.1).

Plasmid construct	Oligo pair no	Oligo sequences	Restriction sites
pASFV_TKend	1	TGAGAATTCAGACCATGGTAGCGGCCG**CGCGTAAGAACGCAGACAAGACG**	*Eco*RI
		TAAAAGCTT**GTATGCTGCGGTAAAGCTGCGTTGAG**	*Hin*dIII
promP72_FFLuc	2	TAAGAATTCTGCAGTCGAC**GGGTCGCCGGAGAAAAGTCAAAAGG**	*Eco*RI
		TGACCATGGTGGCGACCGGT**GCTCCTCCTGATGCCATATATAATGT**	*Nco*I
promU104L_FFLuc_OF	3	TAAGAATTCTGCAGTCGAC**GTGAGGATTTTAATTAGATTTGTG**	*Eco*RI
		TGACCATGGTGGCGACCGGT**GAACCTCATTGTAGTGTTATATTACG**	*Nco*I, *Age*I
promU104L_FFLuc	4	CCGGCCGTGCCGCCAC	
		CATGGTGGCGGCACGG	
promDNApol_FFLuc	5	TAGAGATCT**CAATGGAGATTTGGCGCCGCATG**	*Bgl*II
		TAACCATGG**TCTCAGAACGGTCCATGATAGATATCATC**	*Nco*I
promDNApol+T_FFLuc	6	TAGAGATCT**CAATGGAGATTTGGCGCCGCATG**	*Bgl*II
		TAACCATGG**TCTCAGAACGGTCCATGAATAGATATCATC**	*Nco*I
promCD2v_FFLuc	7	TAGAGATCT**CGCCGCAAGATGTATTCCATTCTC**	*Bgl*II
		TAACCATGG**ATTTTATACACATATATGTTTTA**	*Nco*I
promCD2v_FFLuc_OF	8	TAAGTAGAC**AATTAAATGGTACACTTGCTAATAATG**	*Acc*I
		TAACCATGGT**ATTTTATACACATATATGTTTTATA** *	*Not*I
promP30_FFLuc	9	TAAGACGTC**GTTAGGTGTTGTAACCTTATTGAC**	*Aat*II
		TAACCATGG**ATATATTTAAAATAAAATCCATTC**	*Nco*I
promP30_1_FFLuc	10	TAAGACGTC**TGACTCCATGATCCAGACCGCTAGTC**	*Aat*II
		TAACCATGG**ATATATTTAAAATAAAATCCATTC**	*Nco*I
promP30_2_FFLuc	11	TAAGACGTC**TGTTTCATATTAGAGGGCATC**	*Aat*II
		TAACCATGG**ATATATTTAAAATAAAATCCATTC**	*Nco*I
promP30_3_FFLuc	12	**GATCTCATGGATTTTATTTTAAATATATC**	*Aat*II
		CATGGATATATTTAAAATAAAATCCATGA	*Nco*I
promP72_RenLuc _ATG_1+2_3	13	**CGAAGTCATGGTGGCTAGTCATGGTGGCGA**	
		CCGGTCGCCACCATGACTAGCCACCATGACTT	

which takes around 18 h to be accomplished. The transcription factors or other regulatory elements governing the viral expression schedule are still unknown though. It is believed that this regulation occurs similarly to vaccinia virus, where gene expression is controlled at the level of transcription initiation through a cascade mechanism: transcription factors required for intermediate genes expression are produced as early proteins, factors required for late genes are expressed with intermediate ones, and those required for transcription of early genes are expressed as late proteins and packaged with progeny virions for use upon new infection (reviewed in Broyles, 2003). There are however ASFV genes that are expressed at both early and late phases of infection (Salas et al., 1986; Afonso et al., 1992; Prados et al., 1993; Martins et al., 1994; Rodriguez et al., 1993b). This has also been described for vaccinia virus, with several of its genes being continuously transcribed throughout the infectious cycle through a tandem arrangement of early and intermediate or late promoters preceding the ORF (reviewed in Broyles, 2003). There is scarce knowledge regarding the structure of ASFV promoters though. No consensus sequence is known except for a recognizable increase in A-T rich sequences upstream from an ORF. Only the late phase promoter area from the p72 gene, which encodes the major capsid protein, has been characterized in more detail. A TATA sequence locates close to the mRNA transcription initiation site and the essential promoter sequence is contained within nucleotides-36 to +5 relative to the transcription initiation site, or −40 to +1 relative to the translation start codon (Garcia-Escudero and Vinuela, 2000). Transcription initiation sites have been mapped for a few ASFV genes by primer extension and S1 nuclease protection assays, these locating from approximately −150 to just a few nucleotides upstream the translation initiation codon (reviewed in Rodriguez and Salas, 2013). Transcription termination is usually initiated through a stretch of at least seven thymidylate residues, found in intergenic areas adjacent to the 3′ ends of ORFs (Almazan et al., 1992).

ASFV contains more than 150 ORFs and consequently, since functional viral bi-or multicistronic mRNA molecules have not been described so far, the same number of promoters, provided all deduced ORFs are indeed transcribed. Little is known about the performance of ASFV promoters with regard to their strength and thus efficacy of transcription. The late phase promoter of the p72 gene coding for the major capsid protein, has been - and still is - extensively used to direct e.g. reporter gene expression in recombinant viruses (Garcia-Escudero et al., 1998; Rodriguez et al., 1992, 2004; Andres et al., 2002, 2001; O'Donnell et al., 2016). Current approaches for development of vaccines against ASFV include generation of recombinant viruses expressing duplicates of proteins that are immunogenic or have immunomodulatory functions. For this purpose, viral promoters capable of driving different levels of expression of genes of interest need to be identified. With this aim in mind, we cloned reporter ORFs encoding firefly or renilla luciferase downstream from potential promoter areas from different ASFV genes. The promoter strengths, temporal expression and accuracy were evaluated *in trans* by transient expression from respective expression plasmids, after infection of the transfected cells with ASFV, and *in cis,* by creating recombinant viruses to test for the expression from the viral genome. Our results define ASFV promoter sequences with different strengths, suitable to be used in transient expression experiments and in recombinant ASFV.

2. Materials and methods

2.1. Cell culture

Wild boar lung cells (WSL), kindly provided by Roland Riebe (Collection of Cell Lines in Veterinary Medicine, FLI-Insel Riems), were maintained in a 50:50% (v/v) mixture of Ham's F12 medium and Iscove's modified Dulbecco's medium, pH 7.2, supplemented with 10% fetal bovine serum (FBS), 2,4 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cell cultures were incubated at 37 °C, 5% CO_2 in a humidified atmosphere. In phosphonoacetic acid (PAA) treatments, cell cultures were maintained in medium containing the inhibitor at 0,25 mg/ml throughout infection.

2.2. Plasmid constructs

Oligonucleotide pairs used in construction of the different luciferase