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EXPRESSION OF RECOMBINANT GENES ENCODING FRAGMENTS OF THE PROTECTIVE IMPORTANT PROTEINS OF AFRICAN SWINE FEVER VIRUS IN EUCARYOTIC CELLS

A.R. IMATDINOV, A.D. SEREDA, I.R. IMATDINOV, A.S. KAZAKOVA, O.A. DUBROVSKAYA, D.V. KOLBASOV

All-Russian Institute of Veterinary Virology and Microbiology, Federal Agency of Scientific Organizations, 1, ul. Akademika Bakuleva, pos. Vol'ginskii, Petushinskii Region, Vladimir Province, 601125 Russia, e-mail almazlcf@yandex.ru

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Abstract

Control of African swine fever (ASF) is complicated by the lack of specific prevention medications. The attempts to obtain live attenuated vaccines by conventional methods were not promising, and the inactivated or subunit vaccines have not been developed so far (N.J. Petiska, 1965; D.V. Kolbasov et al., 2014; V. Makarov et al., 2016). The investigation of protective immune response against ASF virus (ASFV) enabled determination of a critical role of cellular defense mechanisms and the most important viral proteins p30, p54 and CD2v (or gp 110-140) involved (P. Gomez-Puertas et al., 1998; J.M. Argilaguet et al., 2012; A.D. Sereda et al., 2015). In view to develop a DNA vaccine against ASFV seroimmunotype 3 we have constructed a set of hybrid plasmids containing fragments of ASFV genes *CP204L*, *E183L* and *EP402R* from attenuated strain MK-200 (pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v). To study expression of the antigenically active polypeptide products for recombinant proteins rp30, rp54 and rCD2v in the eukaryotic cells, we transfected human embryonic kidney cells HEK293T, which stably express the SV40 large T antigen, with recombinant plasmids pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v. By immunoblotting, the polypeptides of the expressed recombinant proteins were identified in the HEK293T cell lysates and characterized for their molecular weights. Regarding size, some antigenically active recombinant polypeptides were as calculated, whereas the other ones apparently resulted from post translational modification. We identified a 21.6 kDa polypeptide after pCI-neo/ASFV/p30 transfection, a major (20.9 kDa) and a minor (36.3 kDa) polypeptides after pCI-neo/ASFV/p54 transfection, and, finally, major polypeptides of 39.8 kDa and 63.1 kDa, together with minor polypeptides of 28.8 kDa and 104.7 kDa when pCI-neo/ASFV/CD2v transfected. These genetic constructions will be helpful to investigate antigenic, immunogenic and protective properties of ASFV recombinant proteins rp30, rp54 and rCD2v.

Keywords: African swine fever, recombinant genes and proteins, transfection, antigenicity

African swine fever (ASF) is a contagious viral septic disease of swine, characterized by fever, toxical signs, hemorrhagic diathesis and high mortality; it may be hyperacute, acute, subacute, chronic and asymptomatic. In acute, the most common form of infection, 100 % of animals die within 5-10 days after onset of clinical signs. The disease affects wild boars and domestic pigs, is transmitted from sick animals and virus carriers through direct contact, alimentary and transplacentally [1]. In the South Eastern Africa, the evolution of ASF virus occurs within the sylvatic cycle, which includes warthogs and ticks of the genus *Ornitodoros* [2, 3]. After outbreaks of ASF reported in Georgia in 2007, the disease has spread to Armenia, Azerbaijan, Nagorno-Karabakh, Iran, Abkhazia, Russia, the Baltic States, Belarus, Poland, and Ukraine [4-6].

The infection control is complicated by the lack of specific prevention.

The attempts to immunize domestic pigs with attenuated strains of ASF in Spain and Portugal in the 1960s were unsuccessful [7, 8]. Later, it was confirmed that due to the high probability of reversion and poor immunobiological characteristics of many attenuated strains the live vaccines based on them were unsuitable for widespread use [9].

The experiments to develop inactivated or subunit vaccines against ASF using conventional methods have also failed because of their inability to induce cell-mediated immunity, which plays a crucial role in the formation of protection at ASF [10-12]. However, the research on the development of safe protective candidate drugs for the temporary protection of swine, ensuring their protection in enzootic areas or the planned slaughter on large pig farms, is ongoing [9, 13]. It has been shown that immunization of swine with recombinant p30 and p54 proteins or a preparation of the GP 110-140 serotype-specific major viral glycoprotein led to partial protection of animals against challenge with homologous virulent isolates [14-17].

The investigation of the protective properties of DNA constructs containing the genes of p30, p54 and CD2v proteins (GP 110-140) suggests a crucial role of the latter in the formation of protection against ASF [18, 19]. Therefore, the research of antigenic and immunogenic characteristics of the expression products of the DNA constructs, encoding genes of the protectively important ASF virus (ASFV) proteins, opens up perspectives for a new generation of drugs against virulent isolates of different immunotypes. The necessary steps of this work are cloning genes in the DNA constructs, their expression in eukaryotic systems, and the confirmation of deriving antigenically active translation products [20]. We believe that attenuated strains of ASF virus, which are characterized by the seroimmunotype and protective properties, should be used as a source of viral genes.

In this work, for the first time we have constructed hybrid plasmids that are suitable for proteomic research and development of the DNA vaccine against ASFV of seroimmunotype III.

Our purpose was to derive DNA constructs containing fragments of the *CP204L*, *E183L*, *EP402R* genes of the ASFV (seroimmunotype III), and identify the corresponding antigenically active translational products of the recombinant rp30, rp54, rCD2v proteins in the transfected eukaryotic cells.

Technique. Mozambique-78 (M-78) and Stavropol 01/08 (highly virulent) ASFV strains of seroimmunotypes III and VIII, as well as the MK-200 strain and the Stavropol 01/08 A_4C_2/9k variant (attenuated) are deposited in the State Collection of Microorganisms (All-Russian Research Institute of Veterinary Virology and Microbiology — VNIIVViM) [21-23].

Protocols to obtain antisera were as follows: No. 1 — twice intramuscular administration of the Stavropol 01/08 A_4S_2/9k attenuated variant at a dose of 10^3 HAU_{50} to a domestic pig (days 0 and 14), intramuscular infection with the Stavropol 01/08 virulent strain at a dose of 10^3 HAU_{50} (day 28) and bleeding the animal (on day 35 after the start of the experiment); No. 2 — blood withdrawal on day 24 after a single intramuscular inoculation of the MK-200 attenuated strain ($10^{6.5}$ HAU_{50}) to a wild boar; No. 3 — a single intramuscular inoculation of the MK-200 attenuated strain at a dose of $10^{6.5}$ HAU_{50} (day 0) to a domestic pig, intramuscular infection with the M-78 virulent strain at a dose of 10^3 HAU_{50} (day 21) and bleeding the animal (on day 35 after the start of the experiment).

The PCR mixture contained the primers, flanking genes *CP204L*, *E183L*, *EP402R* of ASFV MC-200 strain. For the accumulation of PCR products, previously obtained recombinant plasmids pJET1.2/p30-M200/2, pJET1.2/p54-M200/1 and pJET1.2/CD2v-M200/10 were used. Ligation was performed using a commer-

cial kit CloneJET PCR Cloning Kit according to the manufacturer's recommendations (Thermo Fisher Scientific, Inc., USA) and description [24].

Competent cells of the *Escherichia coli* XL-1 strain were transformed by heat shock in the presence of Ca^{2+} ions [25]. Plasmids were isolated from the selected ampicillin-resistant transformants, and the presence of specific inserts was confirmed by the restriction analysis.

Nucleotide sequences of the derived chimerical structures, containing *CP204L*, *E183L* and *EP402R* genes in the pJET1.2 and pCI-neo plasmids, were determined using an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems, Inc., USA).

A human embryonic kidney cell line transformed with the gene of T-antigen of SV40 virus (HEK293T; the Collection of Cell Cultures, VNIIVViM) was transfected with the pCI-neo/ASFV/p30/1, pCI-neo/ASFV/p54/1 and pCI-neo/ASFV/CD2v/1 recombinant plasmids by the calcium-phosphate method according to the guidelines [26], and cultured for 1 to 5 days after the change of the Eagle's MEM (Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products, Russia). Transfection was monitored by the GFP protein expression, with plasmid phMGFP as positive control (Promega, USA) using an Olympus MIT-2 inverted fluorescence microscope (Olympus Corp., Japan). Transfected cells were collected from the substrate mechanically, after thrice washing off from serum proteins with phosphate buffered saline pH 7.2 (FBS), then sedimented by centrifugation (3,000 g, 10 min) and frozen at –70 °C. Further, 10^6 cells were lyzed in 1 cm^3 of RIPA-buffer [24], the cell debris was pelleted by centrifugation (3,000 g, 20 min) and the supernatants were investigated by Western blot analysis. Electrophoresis, electromigration and Western blot analysis were performed by U.K. Laemmle [27], J. Kyhse-Andersen [28] and J.M. Escribano, E. Tabares [29].

The BLASTn program was used to compare the nucleotide sequences of the derived chimerical constructs with those published in GenBank (available on the website http://www.ncbi.nlm.nih.gov/BLAST/). Alignment and analysis were performed using the BioEdit 7.2.5 software (intellectual property of Tom Hall, Freeware) and uGene 1.22 (LLC Novosibirsk Center of Information Technologies UniPro). Prediction of epitopes and structural elements was performed using online servers (Technical University of Denmark, http://www.cbs.dtu.dk/cgi-bin/).

Specific oligonucleotide primers, flanking regions of genes encoding p30, p54 and CD2v proteins

Name	Nucleotide sequence
F-p30Domen	5′-AGTACTGTTAAGTATGATATTGTGAAATCTG-3′
R-p30Domen	5′-AAGTTTAATAACCATGAGTCTTACCACC-3′
F-p54Domen	5′-TCCTCAAGAAAGAAAAAAGCTGCTGCTATTGAG-3′
R-p54Domen	5′-CAAGGAGTTTTCTAGGTCTTTATGCGTATAGG-3′
F-CD2-IgHA	5′-AGTTATAATGAAACAATAATTTTAAATAGTAAT-3′
R-CD2-IgHA	5′-GTGATTTCCTAATAAAAAAGAATATTGATAATA-3′

Results. Bioinformatic analysis of three potentially protective proteins, the p30, p54 and CD2v, of ASFV MK-200 strain revealed the signal sequences, transmembrane regions and potential sites for post-translational modifications. This resulted in identification of extracellular (external to virion) domains of membrane-spanning proteins that carry the highest number of predicted B- and T-cell epitopes in the amino acid sequences.

A clonable region of gene *EP402R* with 49-651 bp (201 residues) excluded native signal and membrane-spanning regions as well as a cytoplasmic domain with 721-1137 bp. Similar regions were localized for *CP204L* (142-546 bp) and *E183L* (160-597 bp) genes. Primers were designed in accordance with the further strategy of "seamless" cloning of DNA constructs by D.G. Gibson et al. [30]. The nucleotide sequences of the specific primers are shown in the Table.

To improve the efficiency of intracellular sorting of these proteins, we used the universal signal elements from heterologous viruses, i.e. the signal se-

quence of fusion (F) glycoprotein of human parainfluenza virus type 1 and a transmembrane region of the HN protein of Sendai virus. To do this, the p30-Domain, p54-Domain and CD2v-IgHA amplicons were subcloned with signal sequences and membrane-spanning regions into the pJET1.2 plasmid vector (Thermo Fisher Scientific, Inc., USA). Based on the PCR screening and restriction analysis, the clones were selected which contained plasmids with the specific nucleotide insertions. Plasmid sequencing showed the integrity of reading frames of the obtained chimerical sequences.

At the next stage, DNA constructs, expressing in eukaryotic cells, were obtained. For this purpose, the corresponding nucleotide sequences were re-cloned into the pCI-neo plasmid vector (Promega, USA) in the NheI and SmaI restriction sites. A CMV promoter in the pCI-neo vector ensures the high expression in eukaryotic systems. The maps of open reading frames of the chimerical genes are shown in Figure 1.

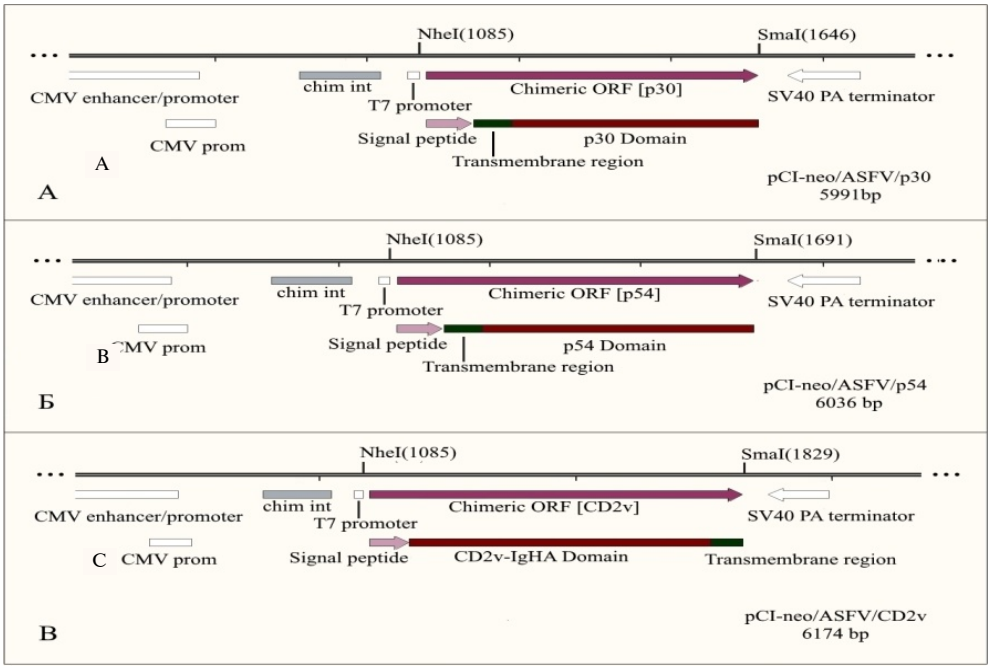

Fig. 1. A diagram of open reading frames for the chimerical genes with cloned sequences of the fragments encoding p30, p54 and CD2v proteins of African swine fever virus in the resulting DNA constructs: A — pCI-neo/ASFV/p30, B — pCI-neo/ASFV/p54, C — pCI-neo/ASFV/CD2v; CMV enhancer/promoter — the human cytomegalovirus immediate early enhancer and promoter, CMV promoter — promoter of human cytomegalovirus, chim intron — a chimerical intron, T7 promoter — promoter of T_7 phage, SV40 PA terminator — the polyadenylation signal and terminator of the SV40 virus DNA, Signal peptide — a signal sequence.

The analysis of the derived recombinant pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v plasmids by PCR with diagnostic primers demonstrated that the amplicons, the sizes of which are consistent with the estimated ones, are synthesized based on these matrices (Fig. 2). The first clones of each construct were chosen for further work.

HEK293T cell line (human embryonic kidney cells transformed with the gene of SV40 virus T-antigen) served as an in vitro expression tool for the obtained recombinant genes. The presence of the virus SV40 replication origin in the pCI-neo plasmid provides for its episomal replication. After by calcium phosphate transfection of the HEK293T cell monolayer using each of the recombinant plasmids (a confluency of 80-90 %) and subsequent culturing for 1-5 days, the trans-

fection efficiency was calculated as a percentage of fluorescent cells from the total number of cells observed with luminescent microscopy. As a control, the same experiments were carried out under identical conditions with the phMGFP plasmid.

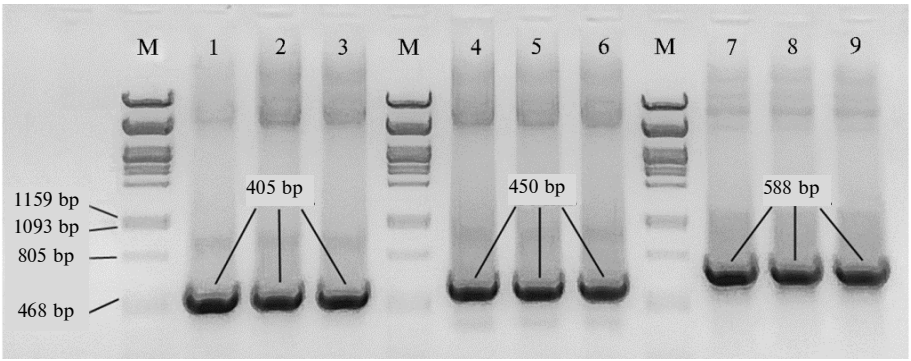

Fig. 2. An electrophoregram of separated PCR products of the chimerical genes in 1.5 % agarose gel: 1, 2, 3 — p30 amplicons (the pCI-neo/ASFV/p30 plasmids derived from *Escherichia coli* were used as a template); 4, 5, 6 — p54 amplicons (the pCI-neo/ASFV/p54 plasmids derived from *E. coli* were used as a template); 7, 8, 9 — CD2v amplicons (the pCI-neo/ASFV/CD2v plasmids derived from *E. coli* were used as a template); M — Lambda DNA/PstI Marker (DNA Sizer 247-11501 bp, GeneOn GmbH, Germany).

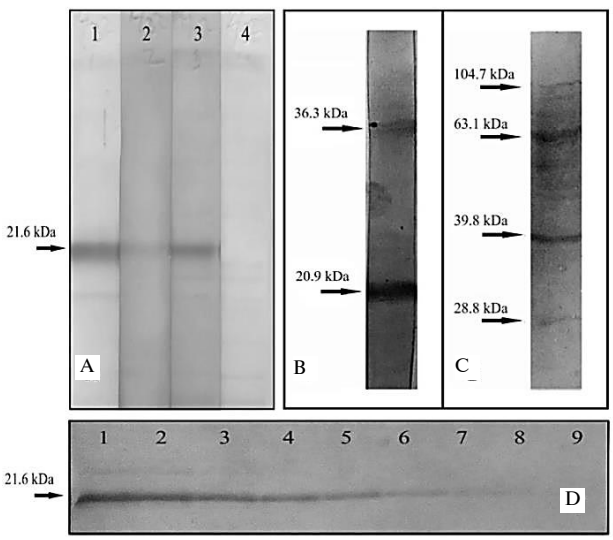

Fig. 3. Western blot analysis showing translation of recombinant rp30 (A), **rp54** (B) **and rp CD2v** (C) **proteins in HEK293T cells transfected with pCI-neo/ASFV/p30/1, pCI-neo/ASFV/p54, pCI-neo/ASFV/CD2v plasmids, and antigenic activity of rp30 protein** (D): 1-4 — numbers of antisera, 5-13 — dilutions (from 1:2 to 1:512 with 2-fold increments) of the lysate of HEK293T cells expressing the rp30 protein. Left arrows indicate the location and molecular weights of virus-specific polypeptides.

The obtained results shown in Figure 3 (A, D) reflecr the antigenic activity of the recombinant (r) rp30 protein which was synthesized in HEK293T cells transfected with the pCI-neo/ASFV/p30/1 plasmid. Western blot analysis with antibodies against ASFV-positive antisera of the domestic pig and wild boar (a 1:20 dilution) showed that its molecular weight is 21.6 kDa. We did not identify virus-specific antigens when testing the blood serum of the intact pig. Western blot analysis of the lysate proteins from the non-transfected HEK293T cells using all antisera gave negative results (data not shown). Titration of a lysate of the HEK293T cells transfected with the pCI-neo/ASFV/p30/1 plasmid (1:2 to 1:512 with 2-fold increments) (see Fig. 3, D) revealed antigenic activity up to a 1:128 dilution, which indicated a high rp30 expression.

Western blot analysis using antiserum No. 3 in a lysate of the HEK293T cells transfected with the pCI-neo/ASFV/p54 plasmid demonstrated the presence of a 20.9 kDa major polypeptide and a 36.3 kDa minor polypeptide (see Fig. 3, B); and if the cells were pCI-neo/ASFV/CD2v transfected, the 39.8 and 63.1 kDa major polypeptides and the 28.8 and 104.7 kDa minor polypeptides were identified (see Fig. 3, C).

The calculated molecular weights of the unmodified recombinant proteins were 21.6 kDa (rp30), 18.7 kDa (rp54) and 28.6 kDa (rCD2v). The Western blot analysis revealed that the actual molecular weights of the obtained recombinant polypeptides matched or were close to the estimated ones, i.e. 21.6 kDa for rp30, 20.9 kDa and 36.3 kDa for rp54 (the latter probably is a rp54 dimer). According to P. Gómez-Puertas et al. [16] and F. Rodriguez et al. [31], the weight of a full-length p54 monomer is 24-28 kDa. In the HEK293T cells transfected with the pCI-neo/ASFV/CD2v plasmid, translated virus-specific polypeptides were 28.8; 39.8; 63.1 and 104.7 kDa. The first of these matched the size of the estimated unmodified molecule rCD2v. The remaining were apparently the forms, which had been variously modified during glycosylation. These results are consistent with the findings from L.C. Goatley and L.K. Dixon [32], who identified in the Vero cells transfected with the SV5CD2vHA plasmid the polypeptides of the recombinant CD2v with molecular weights of 26, 63, 89 and 104 kDa. The authors additionally identified the 42 and 47 kDa polypeptides following the exposure to tunicamycin, a glycosylation inhibitor, or endoglycosidases D and F [32].

Therefore, we obtained DNA constructs with fragments of the *CP204L*, *E183L*, *EP402R* genes of African swine fever virus (ASFV). Western blot analysis using ASFV-specific antisera demonstrated the antigenic activity of the polypeptides expressed as a result of transfecting HEK293T cells with the recombinant pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v plasmids which contained the nucleotide sequences for the recombinant proteins (respectively, for rp30, rp54 and rpCD2v). The next stage of research should determine the immunogenic properties of the recombinant plasmids and improve the obtained gene constructs to develop appropriate protective ones.

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