Alternative Codon Usage of PRRS Virus ORF5 Gene Increases Eucaryotic Expression of GP_5 Glycoprotein and Improves Immune Response in Challenged Pigs

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Running title: Optimized codon usage for PRRSV GP_5 encoding gene

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ABSTRACT

Pigs exposed to GP_5 protein of PRRSV by means of DNA immunization develop specific neutralizing and protecting antibodies. Herein, we report on the consequences of codon bias, and on the favorable outcome of the systematic replacement of native codons of PRRSV ORF5 gene with codons chosen to reflect more closely the codon preference of highly expressed mammalian genes. Therefore, a synthetic PRRSV ORF5 gene (synORF5) was constructed in which 134 nucleotide substitutions were made in comparison to wild-type gene (wtORF5), such that 59% (119) of wild-type codons were replaced with known preferable codons in mammalian cells. *In vitro* expression in mammalian cells of synORF5 was considerably increased comparatively to wtORF5, following infection with tetracycline inducible replication-defective human adenoviral vectors (hAdVs). After challenge inoculation, SPF pigs vaccinated twice with recombinant hAdV/synORF5 developed earlier and higher antibody titers, including virus neutralizing antibodies to GP_5 than pigs vaccinated with hAdV/wtORF5. Data obtained from animal inoculation studies suggest direct correlation between expression levels of immunogenic structural viral proteins and immune response.

Key words: PRRSV; porcine arterivirus; GP_5 protein; codon bias; synthetic gene; expression; adenovirus vectors; immunization.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) was first isolated in the early 1990s and belongs to the *Arteriviridae* viral family in the genus *Arterivirus*, order *Nidovirales* [1]. The viral genome consists of a positive single-stranded RNA molecule of approximately 15 kb in length, composed of nine open reading frames [2-4]. The ORFs 5 to 7 encode the three major structural proteins of virions which are the envelope glycoprotein GP_5 (25-26 kDa), the non-glycosylated membrane protein M (18-19 kDa) and the nucleocapsid protein N (14-15 kDa) [5]. These PRRSV structural proteins are closely associated, GP_5 and M proteins being associated in the form of heterodimers [5].

Circulating antibodies in PRRSV-infected pigs responsible for viral neutralization in cell cultures are mainly directed against GP_5 [6]. Immunization of mice with *Escherichia coli*-expressed GST-ORF5 recombinant fusion protein, as well as with purified PRRSV, induced specific anti-GP_5 neutralizing monoclonal antibodies [7]. Furthermore, genetic immunization of pigs with plasmidic DNA expressing the ORF5 gene triggered the production of transient and low titers of neutralizing antibodies to PRRSV and conferred protection against development of clinical disease and lung lesions but were not sufficient to inhibit virus persistence and shedding in the respiratory tract of PRRSV challenged pigs [8]. On the other hand, when the *E. coli*-expressed GST-ORF5 recombinant fusion protein was used as immunogen prior to challenge with pathogenic virus, the disease was more severe, despite the development of high titers of non-neutralizing antibodies to GP_5 thus suggesting the involvement of a possible antibody-dependent enhancement phenomenon [8]. These findings suggest also that the amounts of GP_5 synthesized in the infected cells, as well as conformation of the protein which may be influenced by the type of oligosaccharide side chains present on the molecule, are apparently crucial to trigger an effective humoral immune response to PRRSV. Since a correlation may exist amongst protection, clinical course of the disease, and seroneutralizing antibody titers [9,10], the present study was designed to increase the efficacy of genetic immunization against antigenic determinants of GP_5.

Live human adenovirus type 5 vector (hAdV) has been shown to be an excellent delivery system for vaccine immunogens [11]. Both replication-defective and replication-competent hAdV have been used as

efficient recombinant vaccines [12] but for biosafety consideration, a replication-defective hAdV is considered to be more suitable for vaccine development. Even if E1 deleted hAdV is unable to replicate *in vivo*, it is able to induce a protective immune response in different species including swine [13]. A synthetic ORF5 gene (synORF5) was then generated in which codon usage was optimized for expression in mammalian cells as previously reported for HIV gp120 [14]. It was demonstrated that synORF5 gene carried by a tetracycline-controlled hAdV is expressed at higher levels than that of the wild-type ORF5 gene (wtORF5) and triggers production of higher titers of neutralizing antibodies which appeared earlier in sera of challenged pigs. This suggests that efficacy of DNA vaccines or recombinant viral vaccines may be improved by optimization of translational process of the GP_5 protein of PRRSV.

MATERIAL AND METHODS

Viruses and cells.

The Québec cytopathogenic IAF-Klop strain of PRRSV was propagated in MARC-145 cells, as previously described [15]. Ad/CMVlacZ [16], a replication-defective E1- and E3-deleted hAdV5, as well as generated hAdVs, were propagated in 293 cells (ATCC CRL-1573) to permit replication of replication-defective hAdVs. AdCMV/tTA permits the constitutive expression of the tetracycline transactivator (tTA) in infected cells using the constitutive CMV immediate-early promoter/enhancer. The tTA is essential to allow expression in hAdV-infected cells of the transgenes which have been cloned downstream and under the control of the tetracycline-regulatable (TR5) promoter. Doxycycline, an analogue of tetracycline, was used at a concentration of 1 μg/ml to inhibit the transgene expression in hAdV-infected cells [16]. The 293 Tet-On cells (Clontech Inc., Palo Alto, CA) are 293 transformed cells that constitutively express the reverse tetracycline transactivator (rtTA). These cells were cultivated in the presence of 1 μg/ml of doxycycline to enhance expression of the transgene placed under the control of the TR5 promoter [16].

Antiserum.

Rabbit monospecific hyperimmune serum (α5) to *E. coli*-expressed ORF5 product of the IAF-Klop PRRSV reference strain was obtained from previous studies [5].

Construction of PRRSV synthetic ORF5 gene.

The codons most frequently used by mammalian cells were used to construct a synthetic ORF5 (synORF5) gene according to Haas et al., (1996) [17]. The DNA template used to construct synORF5 was based on the ORF5 sequence of the IAF-Klop strain of PRRSV (Genbank accession number U64928). The synORF5 gene was assembled by single overlap PCR, as described by Holler et al., (1993) [18].

Generation of recombinant replication-defective hAdVs.

The synORF5 and wtORF5 genes were inserted in the *Bg*l II site of the adenovirus transfer vectors pAdTR5/DC/GFPq so they would be under the control of the TR5 promoter [16]. To facilitate screening and selection of the recombinant hAdVs, the shuttle vector used in these experiments, contains the green fluorescent protein (GFPq) reporter gene downstream the multiple cloning site under the control of an IRES [19].The recombinant plasmids were rescued into the genome of Ad/CMVlacZ, a replication-defective E1- and E3-deleted hAdVs, by homologous recombination in 293 cells, as described elsewhere [15]. The GP_5-expressing hAdVs (hAdV/wtORF5 and hAdV/synORF5) were subjected to three consecutive rounds of plaque purification, then selected viral clones were amplified as previously described [15]. Infectivity titers of the hAdVs were determined by calculation of the plaque forming units (PFU/ml) on 293 cell monolayers, as previously described [15].

Indirect Immunofluorescence assay.

The indirect immunofluorescence (IIF) assay was used to detect the expression of the GP_5 in hAdVs MARC-145 infected cells. Following an incubation period of 24 and 48 h post-infection, those hAdVs infected cells were fixed with a 80% cold acetone solution for 20 min at 4 ^{o}C and the IIF was done using a 1/200 dilution of the α5 serum as previously described [20].

Western blotting experiments.

Sucrose gradient purified-PRRSV was prepared in LB-2 lysis buffer [5], denatured by boiling in the presence of 5% (V/V) β-mercaptoethanol, subjected to 12% SDS-PAGE and electrotransferred onto nitrocellulose membranes as previously described [5,20]. Reactivity of pig sera (dilution 1/50) was tested and the α5 serum was used as a positive control.

Metabolic labeling and immunoprecipitation of PRRSV native or recombinant proteins.

Radiolabeling with [^{35}S]methionine (specific activity of 1,120 Ci/mmole, Amersham Biosciences, Baie d'Urfé, Québec, Canada) of viral proteins synthesized in PRRSV-infected MARC-145 cells, as well

as recombinant proteins synthesized in 293 Tet-On cells infected with hAdVs, was carried out essentially as previously described [15]. Aliquots of 10^7 cpm of clarified radiolabeled lysates of PRRSV-infected, hAdVs-infected or mock-infected cells were incubated with α5 serum and processed as previously described [15].

Animal group description and immunization schedule.

Nine crossbred F1 (Landrace x Yorkshire) castrated specific pathogen-free (SPF) piglets of 4 to 5 weeks of age were randomly divided into one control group and two experimental groups (3 piglets/group). The animals were fed with commercial feed (without tetracycline supplement) and water *ad libitum*. Piglets were given two injections of different mixtures of hAdVs, 32 days apart. Each pig of the first group received 100 µl of a suspension containing 10^9 PFU of hAdV/wtORF5 mixed with 5 X 10^9 PFU of AdCMV/tTA. Each pig of the second group received a suspension mix of 100 µl of 10^9 PFU of hAdV/synORF5 and 5 X 10^9 PFU of AdCMV/tTA. The third group which was used as the control group was given a volume of 100 µl of a suspension containing 5 X 10^9 PFU of AdCMV/tTA alone. These different viral suspensions were prepared in PBS containing 0,02% of the poloxamer SP1017 [21]. The viral suspensions were injected intradermally under the right ear using a 30-gauge needle. The animals were challenged intranasally at day 60 with a dose of 10^5 $TCID_{50}$ of the IAF-Klop strain of PRRSV in 5 ml of clarified cell culture supernatant fluid. Pigs were bled at days 0, 10 and 21 post-challenge.

Virus neutralization and serological tests.

Pig sera were tested for the presence of anti-GP_5 specific antibodies by virus neutralization (VN), IIF, ELISA and WB tests. The VN tests were performed in triplicates with 100 $TCID_{50}$ of the virus/well as previously described [22]. To monitor humoral immune response following challenging of vaccinated pigs, an IIF using PRRSV infected MARC-145 cells as well as a competitive ELISA for detection of antibodies to PRRSV using recombinant *E. coli*-expressed N protein as antigen and a commercial indirect ELISA (IDEXX) for detection of anti-PRRSV antibodies were used as previously described [23].

RESULTS

Construction of a synthetic ORF5 gene based on optimal codon usage.

The synORF5 sequence contains a total of 134 nt substitutions compared to the wtORF5, resulting in an overall 77,8 % (469/603) identity at the nt level, but deduced aa sequences from both wtORF5 and synORF5 are 100 % identical (data not shown). Thus, a new gene coding for the major GP_5 envelope glycoprotein of a North American PRRSV, the IAF-Klop strain, was successfully created, to which EMBL/Genbank accession number AY184209 has been assigned.

Generation of recombinant replication-defective adenoviral vectors.

Our previous results indicate that to be able to obtain replication-defective hAdVs expressing the ORF5 gene, the ORF5 expression has to be regulated because the GP_5 induced cell death by apoptosis and appeared highly toxic per se and subsequently prevent the construction of recombinant hAdV [15]. This is why a regulatable promoter has been used for the construction of hAdVs. Notheworthy, coinfection of the tTA expressing hAdV (AdCMV/tTA) with hAdVs expressing the ORF5 under the control of the TR5 promoter (hAdV/wtORF5 or hAdV/synORF5) was essential for efficient expression of the PRRSV recombinant protein in MARC-145 cells, as well as in 293 cells.

Expression level of the wtORF5 and synORF5.

Following infection at a moi of 100 PFU per cell, until 70 h pi, the intensity of the reporter protein (GFP) fluorescence was higher in cells infected with rec hAdVs expressing the synORF5 gene than those expressing the wtORF5 gene (data not shown). In agreement with the above findings, expression of the GP_5 was also higher in MARC-145 cells infected with hAdV/synORF5 than those infected with hAdV/wtORF5, the intensity of the cytoplasmic fluorescence obtained by IIF using the α5 antibodies being optimal at 48 h pi (figure 1). Accordingly, a significant higher cellular destruction was observed in MARC-145 cells following infection with hAdV/synORF5 due to the higher expression level of the GP_5

(data not shown). Since MARC-145 cells are not permissive to replication of deficient (E1 deleted) recombinant hAdVs, this level of cellular degeneration was rather attributed to the toxicity of the GP_5.

In order to correlate data obtained by IIF with levels of GP_5 synthesis, RIPA experiments were conducted with lysates of 293 rtTA cells infected with either hAdV/wtORF5 or hAdV/synORF5. As shown in figure 2a and b, immunoprecipitation of cell lysates revealed after 48 h pi an increased expression of GP_5 in hAdV/synORF5-infected cells in comparison to the level of GP_5 synthesized in hAdV/wtORF5-infected cells. Densitometry analysis allowed us to determine that the amounts of GP_5 synthesized in hAdV/synORF5-infected 293 rtTA cells or MARC-145 cells corresponded to an increase of 6 to 11 times the amount of the same protein synthesized in hAdV/wtORF5-infected cells. In figure 2a, where the same amounts of cell lysates were used, hAdV/synORF5-infected 293 rtTA cells synthesized 11 times more GP_5 than hAdV/wtORF5-infected cells. Whereas in figure 2b, the amount of GP_5 synthesized in hAdV/synORF5 was 6 times the amount synthesized in hAdV/wtORF5-infected cells, considering that the immunoprecipitation assay was done with 3 times more (in cpm) of hAdV/wtORF5-infected cell lysates.

Antibody response induced in pigs immunized with hAdV/wtORF5 or hAdV/synORF5.

Following two intradermal injections of the mixture of AdCMV/tTA and hAdV/wtORF5, or the mixture of AdCMV/tTA and hAdV/synORF5, none of the vaccinated piglets has developed significant antibody titers as revealed either by ELISA IDEXX (data not shown), IIF (significant titers > 16) or VN (significant titers > 8) (table 1). Furthermore, reactivity to the native GP_5 viral protein could not be demonstrated by Western blotting (WB). Nervertheless, a good antibody immune response, as detected by IIF, could be observed against the hAdV vectors following the first immunization (data not shown). However, following challenge with a low dose of virulent virus given intranasally 28 days after the booster dose, the three pigs vaccinated with hAdV/synORF5 and AdCMV/tTA developed significant antibody titers to the native viral GP_5 protein 10 days post-challenge, as demonstrated by, IIF, VN (table1), WB (figure 3) and indirect ELISA (data not shown). On the other hand, pigs that have been

vaccinated either with hAdV/wtORF5 and AdCMV/tTA, or with the AdCMV/tTA vector alone developed a significant antibody response 10 days post-challenge by IIF and indirect ELISA, but failed to develop significant VN antibody titers at that time (table 1). In the case of the hAdV/wtORF5, a weak reaction to the GP_5 protein was demonstrated by WB only 21 days post-challenge. Therefore only the pigs that had been vaccinated with the hAdV/synORF5 rapidly developed VN antibody titers of 128-256 following challenge (figure 3 and table 1). Also, all hAdV vaccinated groups developed viremia (as tested by RT-PCR) at day 10 post-challenge and one pig in each group still had viremia at day 21 post-challenge (data not shown).

DISCUSSION

We believe that the increase of gene level expression observed in the case of synORF5 compared to the wtORF5 is most likely a purely translational effect, as previously described with HIV-1 [14,17], but still to be proven. Overall, the nt sequence of the synORF5 showed 78 % identity with the wtORF5 nt sequence of the IAF-Klop strain and as 57 % nt identity with the reference European LV strain in comparison to 63% nt identity between the wtORF5 of IAF-Klop strain and LV strain. Nevertheless, deduced aa sequences from both wtORF5 and synORF5 genes of the North American IAF-Klop strain were 100% identical, but displayed only 52 % identity with that of the European strain.

The data presented here clearly support that codon usage can play an important role in determining the expression efficiency in a mammalian cell context as previously described [24]. Also, in regards of improving the immune response, the vaccination of pigs with non-replicative and inducible hAdVs carrying synthetic ORF5 gene with optimized codon usage at least resulted in the establishment of an enhanced immunological memory to antigenic determinants of GP_5, including those involved in the production of neutralizing antibodies. Indeed, following challenge, the hAdV/synORF5-vaccinated pigs developed a higher specific humoral immune response against GP_5, including VN antibodies, than any other vaccinated pigs. Also, the humoral immune response was detected much earlier, being as soon as 10 days post-challenge, indicating that they underwent an earlier anamnestic immune response. The VN antibodies response detected at 10 days post-challenge was the specific consequence of prior immunization with hAdV/synORF5 since normally, VN antibodies are detected after only 3 to 4 weeks in SPF pigs experimentally- or naturally-infected with PRRSV [10,20]. The higher reactivity to GP_5, as well as the higher VN activity of pigs sera that have been vaccinated either with hAdV/wtORF5 or hAdV/synORF5 is in agreement with previous findings indicating that GP_5 is the primary structural glycoprotein of PRRSV involved in virus neutralization activity [6,7,25]. Even if high titers of VN antibodies appeared at day 10 post-challenge, the viremia could not be

prevented (data not shown) as previously observed [10,26,27]. Nevertheless, one report has demonstrated the protective effect of neutralizing antibodies and their potential to prevent viremia [9].

One explanation for the absence of specific immune response prior to challenge inoculation may be the fact that two recombinant hAdVs have to be injected to assure the expression of the transgene. *In vivo*, there is no guaranty that the same cell can be infected by the two hAdVs, thereby reducing the transgene expression compared to the experiments conducted with cell cultures.

A possible explanation for the enhanced immune response in challenged pigs might be the increase in CpG motifs in the synthetic gene that was administered [28] which however appears to be less important than in the case of the syngp120 previously described for HIV-1 [14]. In the last few years, several groups have demonstrated that DNA containing unmethylated CpG motifs is more efficient to trigger B-cell activation [29,30] and also contribute to the immunogenicity of gene vaccines [28,31].

Additional experiments are needed to determine if DNA immunisation with synORF5 also improves the cellular immune response, as described earlier for HIV-1 [14]. Further improvement might also be achieved with cytokine or cytokine-plasmid adjuvants [32-34]. Alternatively, it might be useful to increase the number of potential epitopes by coinjection of DNA plasmids carrying other PRRSV genes, since it has been previously described that epitopes of the GP_4 of European strains are also involved in virus neutralisation [35], and that the M protein is the one mainly involved in the cellular immune response or cellular hypersensibility [36]. Moreover, expression of the two major envelope proteins of EAV as heterodimers was found recently necessary for induction of neutralizing antibodies in mice and horses immunized with recombinant Venezuelan equine encephalitis virus replicon particles [37,38], but this remains to be demonstrated in the case of PRRSV. Nevertheless, our major goal in the very near future is the generation of a hAdV carrying both the synORF5 gene of PRRSV under the control of an inducible promotor for control of the toxicity and the transactivator gene to eliminate the need of using two hAdVs, as described in the present study, and subsequently will improve the transgene expression *in vivo*.

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FIGURES LEGENDS

Fig. 1. Expression of the GP_5 protein in hAdVs MARC-145 infected cells. MARC-145 cell monolayers were co-infected with AdCMV/tTA alone or with hAdV/wtORF5, or with hAdV/synORF5 at a moi of 100 PFU for each viruses. For the detection of the GP_5 expression, cells were fixed with cold acetone and washed twice with PBS, to eliminate spontaneous GFPq fluorescence, after 24 and 48 h pi. Expression of GP_5 of PRRSV was confirmed by specific IIF following incubation in the presence of the α5 serum.

Fig. 2. Radioimmunoprecipitation of GP_5 protein from lysates of 293 Tet-On cells infected with either hAdV/wtORF5 or hAdV/synORF5 at a moi of 1 PFU for each viruses. The immune complexes obtained after incubation of different amounts of total cell lysates in cpm (ratio of 1:1:1 (A) or 3:1:1 (B) as indicated, $1 = 10^7$ cpm) with α5 serum were processed as previously described [15]. After 48 h pi, immunoprecipitation of cell lysates revealed an increased expression of GP_5 in cell cultures that have been infected with hAdV/synORF5 (lane synORF5), in comparison to the level of GP_5 synthesized in cell cultures infected with hAdV/wtORF5 (lane wtORF5). The major structural proteins of the PRRSV (N, M and GP_5), with Mr of 14, 19 and 24-26 kDa, could be immunoprecipitated from lysates of PRRSV-infected cells (lane PRRSV), with no reactivity with lysate of mock-infected cells (mock). ^{14}C-radiolabelled molecular weight size standards (in kDa) were migrated in lane ladder.

Fig. 3. Increased anti-GP_5 humoral immune response of challenged pigs immunized with synORF5 as revealed by Western blot. Four to five -week-old SPF pigs were injected intradermally twice at 32 day-interval with either AdCMV5/tTA alone (tTA), a 1:5 mixture of hAdV/wtORF5 + AdCMV/tTA (wtORF5) or a 1:5 mixture of hAdV/synORF5 + AdCMV/tTA (synORF5), as described in the materials and methods section. They were challenged intranasally four weeks after the boosted injection with 10^5 $TCID_{50}$ of the homologous IAF-Klop strain of PRRSV. Western blot strips were prepared using sucrose-gradient purified PRRSV (IAF-Klop strain) as antigen. The reactivity profiles of pig sera collected 10 days and 21 days post-challenge are illustrated in A and B, respectively. The positive control (+) corresponds to the reactivity of the α5 serum with the sucrose gradient purified virus.

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