Design and construction of African swine fever virus chimeras incorporating foreign viral epitopes

A. Brun[1], **F. Rodríguez**[1,*], **F. Parra**[2], **F. Sobrino**[1,3], and **J. M. Escribano**[4]

[1]Centro de Investigación en Sanidad Animal (CISA-INIA),
Valdeolmos, Madrid, Spain
[2]Departamento de Bioquímica y Biología Molecular, Instituto Universitario de
Biotecnología de Asturias, Universidad de Oviedo, Oviedo, Spain
[3]Centro de Biología Molecular Severo Ochoa (CSIC-UAM),
Cantoblanco, Madrid, Spain
[4]Departamento de Mejora Genética y Biotecnología (INIA),
Madrid, Spain

Summary. In the present work we have studied the feasibility of introducing foreign epitopes into the African swine fever virus (ASFV) particle by genetic manipulation of the virus. For this purpose, we developed specific transfer vectors containing the gene encoding for the highly antigenic structural ASFV protein p54 in which foreign sequences were introduced. DNA sequences encoding continuous linear epitopes, the antigenic site A from foot-and-mouth disease virus (FMDV) VP1 protein and the DA3 antigenic determinant from transmissible gastroenteritis coronavirus (TGEV) nucleoprotein N, were separately cloned into the p54 gene, in a region encoding a non-essential domain of the protein. Chimeric p54 genes were inserted by homologous recombination into the thymidine kinase (TK) locus of ASFV genome. The resulting recombinant viruses efficiently expressed both chimeric proteins under transcriptional control of the p54 promoter, and the chimeric gene products were recognized by antibodies to both p54 and foreign epitopes. The modified p54 proteins were also found in the viral particles and complemented the function of the wild-type p54, since deletion of the p54 gene from recombinant viruses did not affected virus replication in Vero cells. This work demonstrates for the first time the feasibility of incorporating foreign amino acid sequences (up to 18 residues) into a protein component of the ASFV particle without affecting virus viability.

*Present address: Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA, U.S.A.

Introduction

African swine fever virus (ASFV), the sole member of the family *Asfarviridae* (L. Dixon, pers. comm.), is a large DNA virus which infects species of the *Suidae* family and soft ticks of the *Ornithodoros* genus [22, 24]. It was previously shown that the genome of ASFV, a double-stranded DNA molecule, can accept foreign sequences by homologous recombination [17]. This fact was further extended with the construction of plasmid vectors for the generation of deletion mutant viruses or viruses expressing foreign reporter genes [2, 6, 7, 13, 19]. Recently, it was possible to attenuate a virulent strain of ASFV by specific elimination of a viral gene. Animals immunized with the attenuated virus survived the lethal challenge with the homologous virulent virus [26]. The feasibility of manipulating the ASFV genome and its restricted host range makes reasonable to explore the use of ASFV as a swine vector when the incoming information about the genes involved in virulence and pathogenesis allows the stable attenuation of the virus and, consequently, a safer use of such vector.

Previous studies in our laboratory have shown that the ASFV structural protein p54 is an essential protein [19], that localizes in the surface of the virion [3, 19]. Accordingly to its highly immunogenic character, p54 induces neutralizing antibodies during virus infection [8], in agreement with more recent data regarding the functionality of this polypeptide as one of the virus attachment proteins [9]. Furthermore, it has been shown that the p54 gene suffers rearrangements within the coding region without affecting virus viability [18, 21]. These rearrangements produce alterations in the number of copies of the P A A A tetrapeptide present in p54, thus resulting in stepwise modifications in the molecular weight of this protein which are dispensable for virus replication in cell culture.

The above data has favoured the design of a rational strategy to generate ASFV chimeras intended to explore the use of ASFV as a putative virus vector. Here, we have built several recombinant ASF viruses expressing in the surface of the viral particle chimerical p54 proteins containing linear epitopes from two different pathogens: a 18 amino acid epitope corresponding to the immunodominant antigenic site A of foot-and-mouth disease virus (FMDV) VP1 protein [12] and the 12 amino acid DA3 epitope [10] from transmissible gastroenteritis coronavirus (TGEV) nucleoprotein N. Our results indicate that at least 18 amino acid residues can be efficiently inserted in the ASFV particle in a stable form, without affecting virus viability.

Materials and methods

Cells and viruses

Vero cell cultures were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The highly cell culture adapted ASFV isolate BA71V, apathogenic for swine, and the low cell culture passaged attenuated viruses 608V and 1207V, were used in experiments of genetic manipulation.

Transfection-infection of cell cultures

Semiconfluent Vero cell monolayers seeded onto six well culture plates were transfected with 5–10 μg of plasmid DNA per well, using a liposome-mediated transfection procedure [17]. Eight to sixteen hours after transfection cells were washed in DMEM and infected with ASF virus at a multiplicity of infection (m.o.i.) of 1. When the cytophatic effect (c.p.e.) was complete the cultures were harvested, frozen-thawed, and sonicated in ice bath for 1 min. at 80 watts power setting.

Selection and purification of recombinant viruses

Transfection-infection mixtures were used to infect preconfluent Vero cell monolayers seeded onto 100 mm Petri dishes at different dilutions. Two hours post infection the inoculum was removed and cells were covered with 0.4% agarose (Sigma) in DMEM and incubated at 37 °C until viral plaques were observed. X-gal or X-gluc (Biomol) was added to the cultures at a final concentration of 300 μg/ml. Blue plaques were harvested, suspended in DMEM containing 10% FBS and sonicated to disperse the virus from the agarose plugs. The selected viruses were then plaqued for three consecutive rounds of purification in six well dishes.

Plasmid construction

Oligonucleotides 5′GGCCGCCAGTGCACGCGGGGATTTGGCTCACCTAACGACGA-CGCATGCTCGGCATTTGCCGGC 3′ and 5′GGCCGCCCGGCAAATGCCGAG CATGC-GTCGTCGTTAGGTGAGCCAAATCCCCGCGTGCACTGGC 3′ were annealed to generate the nucleotide sequence of the site A from FMDV VP1 protein. Similarly, oligonucleotides 5′ GGCCGCGATCGAAAATTATACAGATGTGTTTGATGACGCGCGGCCTGC 3′ and 5′GGCCGCAGGCTGTGTGTCATCGGGCGCGTCTGTATAATTTTCGATCGC 3′ were annealed to generate the nucleotide sequence corresponding to the DA3 epitope from TGEV nucleoprotein N. The annealed oligomers were then ligated to NotI digested plasmid pINS β-gus p54 [19]. The resulting plasmids were named pINS β-gus p54/siteA and pINS β-gus p54/DA3 respectively.

The construction of plasmid pΔp54, designed to inactivate the wild type copy of the p54 gene, has been previously described [19]. Briefly, a 862 bp PCR product containing the ORF and flanking sequences from p54 gene was cloned into pUC19 vector. The resulting plasmid, pUCp54, was then digested with AccI and NotI in order to eliminate 322 bp from the p54 ORF, blunt ended with Klenow enzyme, dephosphorylated and ligated to a 3.3 kb fragment, containing a 200bp late ASFV p72 protein promoter region sequence immediately upstream the β-gal gene, that was obtained by blunt ending the XbaI digested plasmid pINS β-gal.

Western blot and immunoblotting

Infected Vero cell extracts or purified virus were subjected to electrophoresis in 15% polyacrylamide gels and electrotransferred to nitrocellulose sheets. Nitrocellulose filter strips were blocked with 2% non-fat dried milk in phosphate buffered saline (PBS) for 30 min. at room temperature and incubated either with anti-p54 polyclonal serum raised against a baculovirus-expressed p54 [8], monoclonal antibody SD6, which recognizes VP1 site A in type C_1 FMDV [11] or monoclonal antibody DA3, which recognizes TGEV nucleoprotein N [10]. Strips were then washed extensively and incubated with a rabbit anti-mouse IgG conjugated with peroxidase. Immunocomplexes were detected either by chromogenic or chemiluminescent reactions.

Purification of viral particles and analysis of recombinant virus DNA

Viral particles from infected Vero cell culture supernatants were purified in Percoll gradients [4]. For the isolation of viral DNA, ASFV infected Vero cells were incubated with 500 µg/ml proteinase K in 50 mM Tris·Cl pH 7.8, 0.5% SDS, 6% (w/v) sucrose. DNA was phenol extracted, ethanol precipitated and digested with *Eco* RI restriction endonuclease. The resulting DNA fragments were resolved by agarose gel electrophoresis, and then blotted onto uncharged Nylon membranes (Amersham). The membranes were probed with a digoxigenin-11-dUTP labeled 322 bp DNA fragment corresponding to the region replaced by insertional inactivation of wild-type p54 gene [19].

Growth curve of recombinant viruses

Approximately 3.5×10^4 cells were infected at a moi of 1 with either parental viruses BA71V, 608V and 1207V, with double recombinant viruses BA71V and 608V (GUS$^+$/GAL$^+$) or with single recombinant 1207V (GUS$^+$) virus. Viral growth was then monitored by plaque assay of viral yields at 0, 8, 16, 24, 48 and 72 h post infection (hpi). Viral inocula were seeded onto Vero cells in 96 well plates and layered with 3% carboximethylcellulose (Sigma) in DMEM supplemented with 5% FBS. The layer was removed by vacuum aspiration and then viral plaques were immunostained [25] using monoclonal antibody 3F8, which recognizes ASFV protein p30. Input virus, defined as number of plaque forming units adsorbed to the cells one hour after infection, was considered as viral titer at 0 hpi.

Results

Construction of ASFV/foreign epitope chimeras

The study of p54 heterogeneity proved that differences in size displayed after adaptation of ASFV to growth in cell culture [1] consisted of changes in the number of copies of an imperfect direct repeat formed by 12 nucleotides coding for a PAAA tetrapeptide [18, 21]. Wild type and low passage viruses contained only two repeats while one to more than four copies were found in highly passaged viruses. These alterations in the number of PAAA blocks produced stepwise changes in the electrophoretic mobility of p54. Nevertheless, all p54 species were functional since they were specified by single virus clones and incorporated to the viral particle without affecting virus morphogenesis [18]. We took advantage of the occurrence of these dispensable repetitions to introduce foreign sequences between two consecutive PAAA motifs. The coding sequence for the three alanine residues provided for a NotI restriction endonuclease recognition site, thus facilitating the insertion of foreign sequences into the p54 coding region (Fig. 1). Following this approach we generated plasmids containing modified p54 genes including either the site A epitope from FMDV VP1 or the DA3 epitope from TGEV nucleoprotein N. Subsequent generation of recombinant viruses with modified p54 proteins was achieved in a two-step process (Fig. 2) as reported [19]. The first step involved the insertion of the chimeric p54 gene together with a reporter gene, β-glucuronidase (β-*gus*), into the thymidine kinase locus of the ASFV genome. In the second step, the wild-type copy of the p54 gene was disrupted by the insertion of a second reporter gene, β-galactosidase (β-*gal*), into the genome of β-gus expressing viruses, generated after the first homologous recombination.

```
          Not I        Not I
1..ACTGGCGGGCCAGCGGCCGCCACCTGCGGCCGCCGAGTGCTCAT..552 bp    P54 wild type
   T  G  G  P  A  A  A  A  P  A  A  A  A  S  A  H

                Not I
1...ACTGGCGGGCCAGCGGCCGCCGATCGAAAATTATACAGATGTGTTTGATGACACAGCCTGCGGCCGCCGAGTGCTCAT...588 bp    P54/DA3
    T  G  G  P  A  A  A  I  E  N  Y  T  D  V  F  D  D  T  Q  P  A  A  A  S  A  H

          Not I                                                                           Not I
1...ACTGGCGCGGCCAGCGGCCGCCGCCAGTGCACGCGGGGGATTTGGCTCACCTAACGACGACGCATGCTCGGCATTTGCCGGCCGCCGAGTGCTCAT...603 bp    P54/siteA
    T  G  G  P  A  A  A  A  S  A  R  G  D  L  A  H  L  T  T  T  H  A  R  H  L  P  A  A  A  S  A  H
```

Fig. 1. Strategy of sequence insertion in the p54 gene. Foreign nucleotide sequences are represented in boldface type and the corresponding amino acid sequences have been underlined. Italic letters show the specific amino acid sequences for the introduced viral epitopes
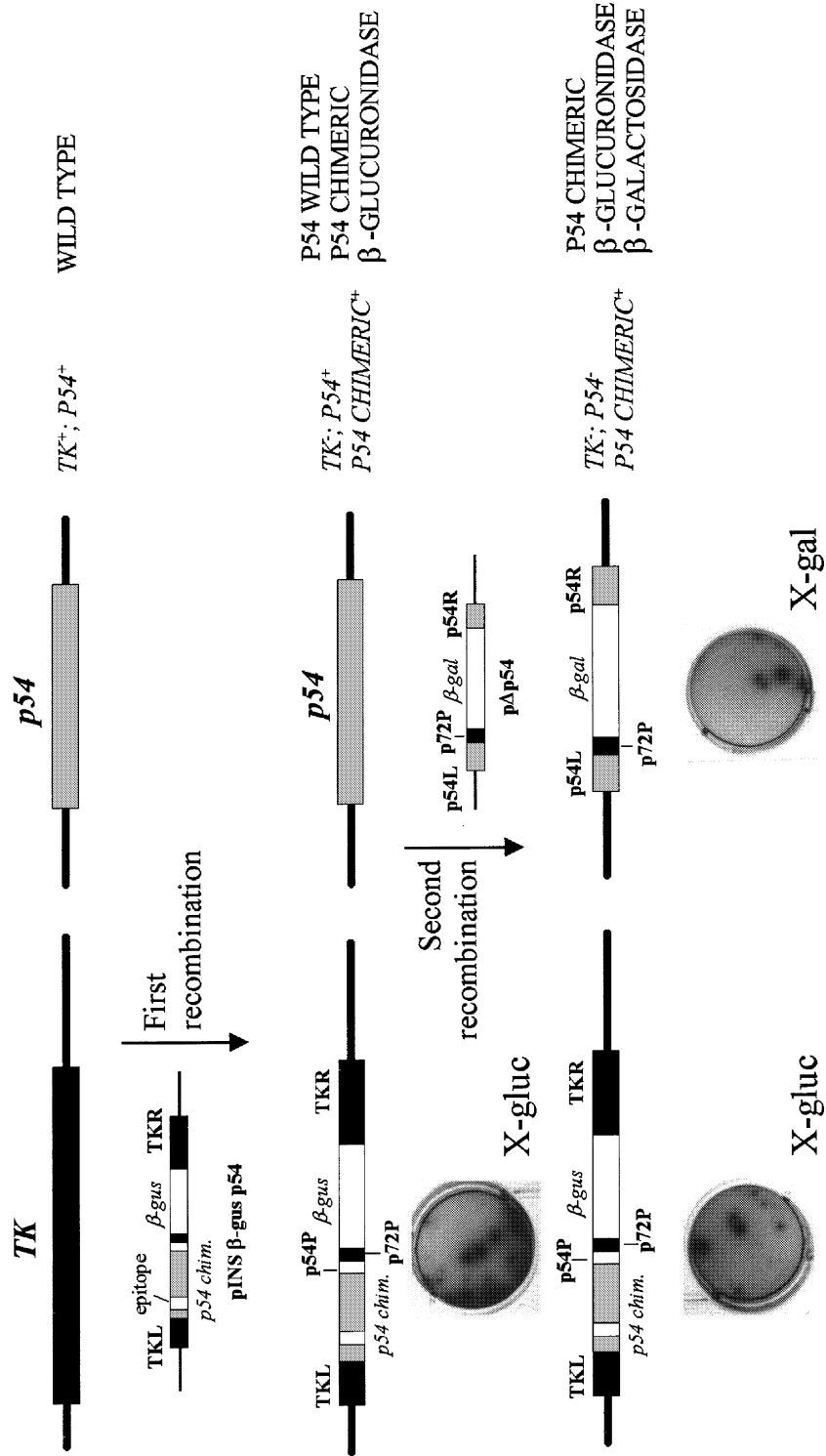
A. Brun et al.

**Fig. 2.** General outline for the generation of chimeric ASF viruses

The method generates two types of recombinant viruses, either with double or single copies of p54 and with one or two reporter proteins, as a consequence of single or double recombination events, respectively. By this methodology, we obtained single and double recombinant Ba71V and 608V viruses, containing the epitope from FMDV virus defined by monoclonal antibody SD6, a single and double recombinant Ba71V virus containing DA3 epitope, and a single recombinant 1207V virus containing DA3 epitope.

Expression of chimeric p54/foreign epitope proteins

In order to detect the expression of p54 chimeric proteins in Vero cells infected with recombinant viruses, we carried out Western blot assays using infected cell extracts and specific antibodies to both p54 and the viral epitopes inserted in the p54 locus. An inmunorreactive band of approximately 26 kDa corresponding to chimeric protein p54/site A was readily detected using both specific anti-p54 serum and monoclonal antibody SD6 (Fig. 3B). Thus, incorporation of the 18 amino acid site A epitope generated a p54 chimeric protein that increased in 1 kDa the relative molecular mass observed in wild-type ASFV-infected cells. In contrast, incorporation of the 12 amino acid DA3 epitope from nucleoprotein N into p54 increased the relative molecular mass of the wild-type protein in about
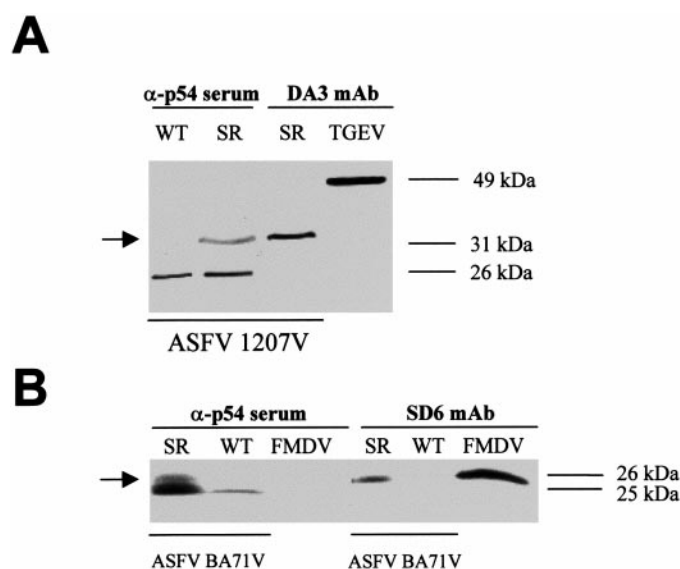
Fig. 3. Western blot analysis of chimeric p54 proteins. **A** Detection of p54/DA3 with monospecific polyclonal anti-p54 serum or with monoclonal antibody DA3 directed to N protein of TGEV. *WT* 1207V wild-type infected Vero cell extracts; *SR* 1207V/DA3 single recombinant virus; *TGEV* TGEV infected ST cell extracts. **B** Detection of p54/Site A with monospecific polyclonal anti-p54 serum or with monoclonal antibody SD6 directed to FMDV antigenic site A (26 kDa). *WT* BA71V wild-type infected Vero cell extracts; *SR* BA71V/Site A single recombinant virus; *FMDV* purified FMDV. The arrows show the position of the chimerical p54 proteins

A   **B**   **C**
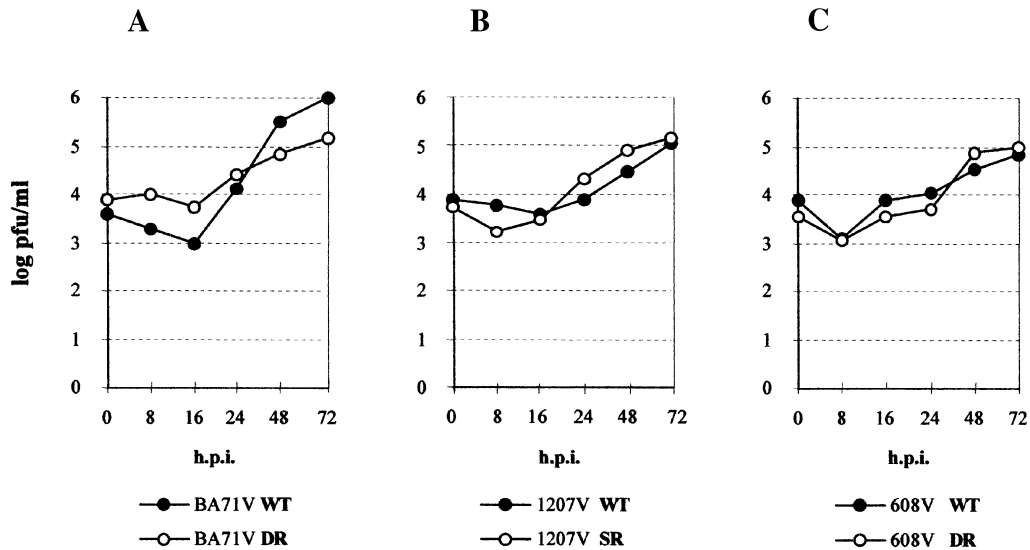
Fig. 4. Analysis of the replicative phenotype of genetically manipulated viruses in Vero cells. **A** Parental (*WT*) and double recombinant (*DR*) BA71V/Site A viruses. **B** Parental (*WT*) and single recombinant (*SR*) 1207V/DA3 viruses. **C** Parental (*WT*) and double recombinant (*DR*) 608V/Site A viruses

6 kDa, generating a chimerical p54/DA3 polypeptide of approximately 31 kDa (Fig. 3A). These differences in molecular mass of p54 after incorporation of foreign epitopes were not due to incorporation of repetitive copies of DA3 epitope into the p54. Sequence analysis of the plasmid constructs used for homologous recombination with the parental viruses demonstrated that only one copy of the sequence encoding for each epitope was inserted into the p54 gene. Postransductional modifications of chimeric p54 proteins incorporated to the ASFV genomes, that may account for the altered molecular mass observed with DA3 epitope, were not analyzed. Importantly, the antigenicity of p54 after incorporation of the foreign epitopes was not substantially altered since a monospecific antiserum reacted with chimeric proteins similarly as it did with wild-type p54.

To assess whether the growth rate of the recombinant viruses was altered by the manipulation of the original p54 gene, a growth curve analysis was performed both with single (1207V/DA3) and double recombinant viruses (Ba71V/Site A and 608/Site A) and referred to their parental viruses. Viral growth was not substantially affected by the insertion of neither the TGEV nor the FMDV epitopes (Fig. 4).

Genomic analysis of recombinant viruses

To establish whether the genomic structure of the recombinant viruses was as predicted, DNA was isolated from Vero cell cultures infected either with recombinant or parental viruses. Total DNA was digested with EcoRI and analyzed by Southern blotting. Membranes containing transferred DNA were probed with
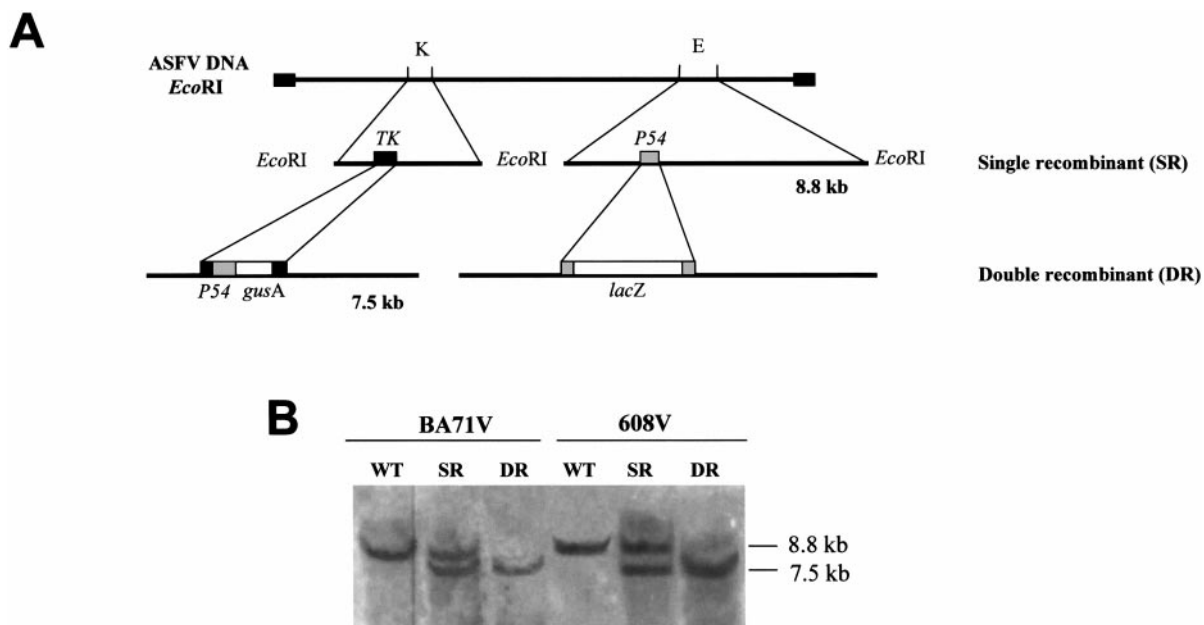
Fig. 5. Genomic analysis of single and double recombinant BA71V/Site A and 608V/Site A viruses. **A** Schematic representation of the wild-type ASFV genome indicating the position of the EcoRI K and E fragments and the predicted genomic structure of the single (*SR*) and double (*DR*) recombinant viruses. **B** Southern blot analysis of parental (*WT*) single (*SR*) and double (*DR*) recombinant BA71V/Site A and 608V/Site A virus genomes using a probe consisting on a digoxigenin-11-dUTP labeled 322 bp DNA fragment corresponding to the region replaced by insertional inactivation of wild-type p54 gene

a digoxigenin-11-dUTP-labeled internal Acc I/Not I p54 gene fragment, the same gene region deleted by the insertion of β-gal gene. The results obtained were consistent with the predicted genomic structure for the recombinant viruses (Fig. 5). As expected, the p54 probe hybridized, both in parental and single recombinant viruses, to a 8.8 kb fragment corresponding to the ASFV DNA EcoRI E restriction fragment, and to a 7.5 kb fragment, corresponding to an altered EcoRI K fragment, in single and double recombinant viruses.

Detection of inserted epitopes in the viral particle

Since p54 has been found as a component of the virion structure [3, 8, 18, 21], we performed experiments to check whether the chimeric p54 proteins effectively incorporated to the viral particle. For this purpose Percoll purified preparations of single and double recombinant BA71V/DA3 viruses, containing both the chimeric and wild-type copies of the p54 protein or only the chimeric p54 respectively, were subjected to SDS-PAGE and analyzed by Western blotting using a monospecific anti-p54 serum (Fig. 6). Antibodies were able to detect the wild-type (25 kDa) and chimeric (31 kDa) polypeptide incorporated to viral particles, indicating that both forms of p54 were efficiently incorporated to the virions.

α-p54 serum

DR SR

P54/DA3 →

— 31 kDa

— 26 kDa
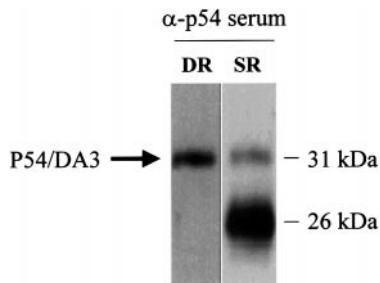
Fig. 6. Western blot analysis of chimeric p54/DA3 protein incorporated to the viral particle. Percoll purified single recombinant (*SR*) or double recombinant (*DR*) BA71V/DA3 viruses presenting two or only one copy of p54 forms incorporated to the viral particle, respectively

Discussion

Infection with attenuated ASFV strains produces a strong cellular and humoral immune responses in pigs [23], conferring in some cases a prolonged immunity protecting for years against homologous virulent infection [14, 20]. Recent studies about ASFV genes involved in virulence and/or pathogenesis allowed for the attenuation of a virulent ASFV or modifications in pathogenesis by obtention of recombinant deletion mutants [2, 26]. These genetically modified attenuated viruses may serve not only as ASFV vaccines but as a potential swine vectors to trigger potent immune responses against foreign proteins or epitopes expressed during ASFV infection, producing high titer systemic and secretory antibodies. In addition ASFV may have practical uses for in vitro studies of T-cell epitope mapping of foreign antigens since this virus infects monocyte/macrophages and other antigen presenting cells [5, 15, 16].

Genetic manipulation of ASFV can be achieved by homologous recombination with a similar efficiency to that routinely obtained for vaccinia virus [17]. Therefore, and as a first step in the development of an ASFV derived vector, we have designed a method to incorporate foreign sequences into the ASFV particle. Three ASFV strains were genetically modified to produce chimeric p54 proteins carrying two different foreign viral epitopes. These chimeric proteins were expressed in cells infected by recombinant viruses at lower level than wild-type p54. This difference in protein expression could be due to the localization of the gene copy in the virus genome, since chimeric p54 was expressed under the control of the same promoter than original p54. Chimeric p54 proteins were efficiently incorporated to the virus particle either when the virus expressed both wild-type and chimerical p54 protein simultaneously or the modified version of the protein alone. The proportion of p54 form incorporated to the virus particle was proportional to their relative abundance in infected cells. The generated recombinant viruses were stable at least after 10 consecutive passages in cell culture and did not present significant alterations in virus yields with respect to parental viruses.

In this study we have inserted an epitope of 18 amino acid residues (antigenic site A) into the p54 protein without affecting significatively the virus morphogenesis and the replication phenotype of the chimeric virus. Since subpopulations with p54 proteins of more than 35 kDa have been described [1], corresponding approximately to a 40 amino acids repetition [18], the possibility of introducing

foreign sequences of more than 20 amino acids containing multiple epitopes remains opened and should be further studied. In addition, several copies of chimeric p54 proteins containing foreign epitopes from different swine pathogens could be probably incorporated into the same viral particle, since wild-type and chimeric p54 proteins were incorporated to the virions in single recombinant viruses. Finally, since p54 is one of the most antigenic ASFV proteins, it is reasonable to speculate about the possibility that cloned epitopes could enhance its own antigenicity when they are associated with p54.

Acknowledgements

References

1. Alcaraz C, Brun A, Ruiz-Gonzalvo F, Escribano JM (1992) Cell culture propagation modifies the African swine fever replicative phenotype in macrophages and generates viral subpopulations differing in protein p54. Virus Res 23: 173–182
2. Borca MV, Carrillo C, Zsak L, Laegreid WW, Kutish GF, Neilan JG, Burrage TG, Rock DL (1988) Deletion of a CD2-like gene, 8-DR, from African swine fever virus affects viral infection in domestic swine. J Virol 72: 2 881–2 889
3. Brookes SM, Sun H, Dixon LK, Parkhouse RM (1998) Characterization of African swine fever virion proteins j5R and j13L: immuno-localization in virus particles and assembly sites. J Gen Virol 79: 1 179–1 188
4. Carrascosa AL, del Val M, Santarén JF, Viñuela E (1985) Purification and properties of African swine fever virus. J Virol 54: 337–344
5. Casal I, Enjuanes L, Viñuela E (1984) Porcine leukocyte cellular subsets sensitive to ASFV in vitro. J Virol 52: 37–46
6. García R, Almazán F, Rodríguez JM, Alonso M, Viñuela E, Rodríguez JF (1995) Vectors for the genetic manipulation of African swine fever virus. J Biotechnol 40: 121–131
7. Gómez-Puertas P, Rodríguez F, Ortega A, Oviedo JM, Alonso C, Escribano JM (1995) Improvement of African swine fever virus neutralization assay using recombinant viruses expressing chromogenic marker genes. J Virol Methods 55: 271–279
8. Gómez Puertas P, Rodríguez F, Oviedo JM, Ramiro-Ibáñez F, Ruiz-Gonzalvo F, Alonso C, Escribano JM (1996) Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. J Virol 70: 5 689–5 694
9. Gómez-Puertas P, Rodríguez F, Oviedo JM, Brun A, Alonso C, Escribano JM (1998) The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. Virology 243: 461–471
10. Martín Alonso JM, Balbín M, Garwes DJ, Enjuanes L, Gascón S, Parra F (1992) Antigenic structure of transmissible gastroenteritis virus nucleoprotein. Virology 188: 168–174
11. Mateu MG, Rocha E, Vicente O, Vayreda F, Navalpotro C, Andreu D, Pedroso E, Giralt E, Enjuanes L, Domingo E (1987) Reactivity with monoclonal antibodies of viruses from an episode of foot-and-mouth disease. Virus Res 8: 261–274
12. Mateu MG, Martínez M A, Rocha E, Andreu D, Parejo J, Sobrino F, Domingo E (1989) Implication of a quasispecies genome structure: effect of frequent, naturally occurring

amino acid substitutions on the antigenicity of foot-and-mouth disease virus. Proc Natl Acad Sci USA 86: 5 883–5 887

13. Neilan JG, Lu Kutish GF, Zsak L, Lewis TL, Rock DL (1997) A conserved African swine fever virus Iκ-B homolog, 5EL, is non-essential for growth in vitro and virulence in domestic pigs. Virology 235: 377–385

14. Onisk DV, Borca MV, Kutish G, Kramer E, Irusta P, Rock DL (1994) Passively transferred African swine fever virus antibodies protect swine against lethal infection. Virology 198: 350–354

15. Ramiro-Ibáñez F, Escribano JM, Alonso C (1995) Application of a monoclonal antibody recognizing protein p30 to detect African swine fever virus infected cells in peripheral blood. J Virol Methods 55: 339–345

16. Ramiro-Ibáñez F, Ortega A, Brun A, Escribano JM, Alonso C (1996) Apoptosis: a mechanism of cell killing and lymphoid organ impairment during acute African swine fever virus infection. J Gen Virol 77: 2 209–2 219

17. Rodríguez JM, Almazán F, Viñuela E, Rodríguez JF (1992) Genetic manipulation of African swine fever virus: construction of recombinant virus expressing the β-galactosidase gene. Virology 188: 67–76

18. Rodríguez F, Alcaraz C, Eiras A, Yáñez RJ, Rodríguez JM, Alonso C, Rodríguez JF, Escribano JM (1994) Characterization and molecular basis of heterogeneity of the African swine fever virus envelope protein p54. J Virol 68: 7 244–7 252

19. Rodríguez F, Ley V, Gómez-Puertas P, García R, Rodríguez JF, Escribano JM (1996) The structural protein p54 is essential for African swine fever virus viability. Virus Res 40: 161–167

20. Ruiz-Gonzalvo F, Carnero ME, Bruyel V (1983) Immunological responses of pigs to partially attenuated African swine fever virus and their resistance to virulent homologous and heterologous viruses. In: Wilkinson PJ (ed) FAO/CEC Expert consultation in African Swine Fever Research Sardinia, pp 206–216

21. Sun H, Jacobs SC, Smith GL, Dixon LK, Parkhouse RM (1995) African swine fever virus gene j13L encodes a 25–27 kDa virion protein with variable numbers of amino acid repeats. J Gen Virol 76: 1 117–1 127

22. Viñuela E (1985) African swine fever virus. Curr Top Microbiol Immunol 116: 151–170

23. Wardley RC, Norley SG, Martins CV, Lawman MJP (1987) The host response to African swine fever virus. Prog Med Virol 34: 180–192

24. Wilkinson PJ (1989) African swine fever virus. In: Pensaert MB (ed) Virus infection of porcines. Elsevier, Amsterdam, pp 17–37

25. Zsak L, Onisk DV, Afonso CL, Rock DL (1993) Virulent African swine fever virus isolates are neutralized by swine serum and by monoclonal antibody recognizing a 72 kDa viral protein. Virology 196: 596–602

26. Zsak L, Caler E, Lu Z, Kutish GF, Neilan JG, Rock DL (1997) A nonessential African swine fever virus gene UK is a significant virulence determinant in domestic swine. J Virol 72: 1 028–1 035

Authors' address: Dr. J. M. Escribano, Departamento de Mejora Genética y Biotecnología, INIA, Ctra. de La Coruña, Km7, 28040 Madrid, Spain.