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The structural protein p54 is essential for African swine fever virus viability

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Abstract

Protein p54, one of the most antigenic structural African swine fever virus (ASFV) proteins, has been localized by immuno-electron microscopy in the replication factories of infected cells, mainly associated with membranes and immature virus particles. Attempts to inactivate the p54 gene from ASFV by targeted insertion of β-galactosidase selection marker was uniformly unsuccessful, suggesting that this gene is essential for virus viability. To demonstrate that, we inserted in the TK (thymidine kinase) locus of the virus a construction containing a second copy of the p54 gene and β-glucuronidase selection marker under the control of p54 and p73 promoters, respectively. Virus mutant clones expressing a second copy of p54 and β-glucuronidase were used to achieve deletion mutants of the original copy of the gene. Virus mutants expressing only the second inserted copy of p54 and the two selection markers mentioned above were successfully obtained. Therefore, we have demonstrated that the p54 gene product plays an essential role in virus growth, characterizing for the first time in ASFV an essential virus gene.

1. Introduction

African swine fever virus (ASFV) causes an important disease of domestic pigs and related species of the suidae family. The ASFV genome consists of a single molecule of double-stranded DNA of about 170 kb. This molecule shares several structural features with the DNA of poxviruses, such as the presence of hairpin loop structures at the DNA ends (González et al., 1986) and terminal inverted repeats (Almendral et al., 1984). ASFV replicates within the cytoplasm of the infected cells and induces ~100 polypeptides both in pig macrophages, the natural host cells (Alcaraz et al., 1992), or in different cell lines (Santarén and Viñuela, 1986). About 40 of these polypeptides have been described as being incorporated into the viral particle (Carrascosa et al., 1985; Esteves et al., 1986). One of the most antigenic viral structural proteins during infection, the p54, has been characterized (Rodríguez et al., 1994). This protein is expressed at late times, and has been shown to be involved in the generation

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of viral diversity during cell culture propagation (Alcaraz et al., 1992; Rodríguez et al., 1994).

At present, the relevance of the different ASFV proteins in virion morphogenesis is unknown. Previously, non-essential genomic regions and genes specific for ASFV viability have been characterized (Agüero et al., 1990; Blasco et al., 1989a; Rodríguez et al., 1992; Rodríguez et al., 1993; Santurde et al., 1988). This study mainly concerns the characterization of p54 as an essential gene for ASFV viability.

2. Methods

For further characterization of protein p54 we analyzed the location of this antigen in infected cells and viral particles by immuno-electron microscopy. Infected Vero cells were removed from the culture dish by proteinase K (20 μg/ml) treatment on ice for 2–3 min and fixed in 8% paraformaldehyde in 0.25 M Hepes pH 7.4 overnight. Cell pellets were infiltrated with 2.1 M sucrose in PBS, frozen and stored in liquid nitrogen. Ultrathin sections were cut at −90°C, transferred to formvar and coated grids and then incubated with 10% FCS in PBS as a blocking agent before antibody incubation. Grids with sections were incubated with a swine antisera against baculovirus expressed p54 or *E. coli* expressed p72 as control, and recognizing only these proteins in radioimmunoprecipitation and Western blot, respectively (unpublished results). These sera were employed diluted 1:100 in PBS containing 5% FCS. Finally, the immunocomplexes were revealed with protein A-gold conjugate (10-nm) diluted in PBS with 10% FCS and then washed with PBS and water prior to staining with uranyl acetate as described (Griffiths, 1993).

3. Results

Fig. 1 shows immunolabelling of a section of a viral factory with different stages of viral assembly. Viral particles surrounded by one envelope were heavily labelled with anti-p54 antiserum (Fig. 1A and Fig. 1B), while anti p72 antiserum, used as control, labelled mainly more mature, hexagonal-shaped, viral particles surrounded probably by an additional envelope (Fig. 1C). Other differentially shaped membrane structures were also labelled with anti-p54 antibodies (Fig. 1A). The p54 antigen could not be easily detected by immunolabelling in the mature viral particles (Fig. 1A). Sections of ASFV-infected cells at other times post-infection showed similar localization for p54. Uninfected cells processed as ASFV-infected cells and incubated with anti-p54 or p72 sera did not show labelling of any cellular structure (data not shown).

Recently, the feasibility of genetically manipulating the genome of ASFV growing in cell cultures has been assessed (Rodríguez et al., 1992; Rodríguez et al., 1993; Gómez-Puertas et al., 1995), showing that it is possible to either insert or delete DNA sequences from the viral genome by homologous recombination during the process of viral replication. In order to demonstrate the essentiality of the p54 gene for virus viability we designed specific vectors to analyze whether p54 gene could be deleted from the virus genome.

The ASFV strain BA71V, propagated and titrated as described (Enjuanes et al., 1976), was used to obtain mutants by homologous recombination in Vero cells. Transfections of Vero cells were carried out as described previously for ASFV (Rodríguez et al., 1992), using a liposome-mediated transfection protocol (Felgner et al., 1987). Preconfluent cell monolayers in 30-mm plastic Petri dishes were transfected with 5 μg of each plasmid DNA without carrier DNA. Eighteen h after transfection, the cell cultures were washed 3 times with 5 ml of Dulbecco's modified Eagle's medium and then infected with ASFV (MOI 1). Purification of plasmid DNA, endonuclease restriction analysis, DNA cloning, polymerase chain reaction (PCR), Southern blotting, and preparation of radioactive probes were performed using standard protocols (Sambrook et al., 1989).

The construction of the p54 deletion vector pΔp54 involved several steps summarized in Fig. 2A. A 1862-bp *Eco*RI-*Pst*I fragment containing the complete p54 ORF and flanking regions was obtained by PCR using the primers 5′-GCGC-

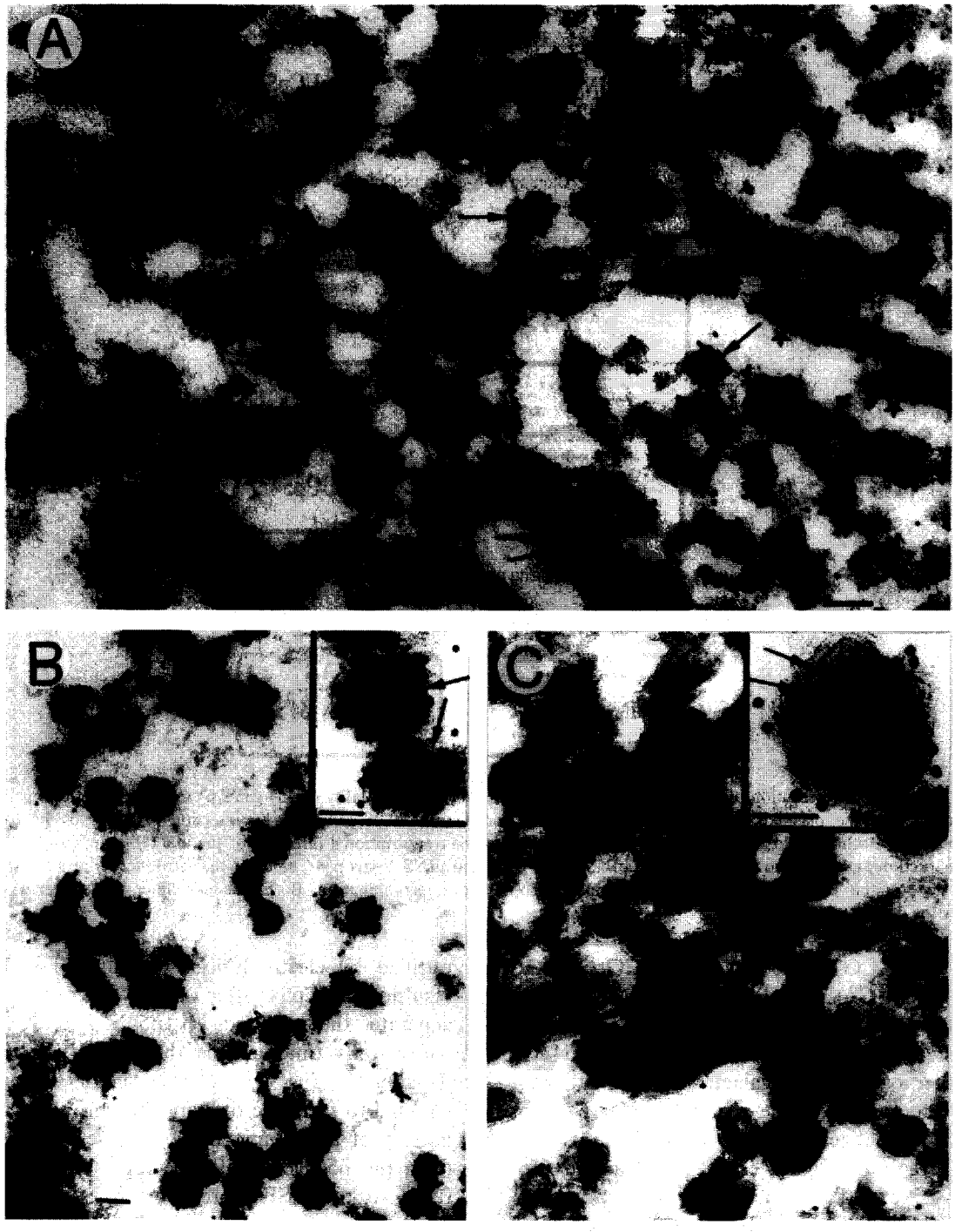

Fig. 1. Immunolabelling of a section of a viral factory with different stages of viral assembly. Ultra-thin cryosections of BA71V ASF virus-infected Vero cells at 24 h post-infection were labelled with anti-p54 (A and B) or anti-p72 (C) antibodies and then with 10-nm protein A-gold. The distribution of p54 was mainly in immature viral particles surrounded by one envelope (A and B), while the distribution of p72 was mainly in more mature particles with additional envelopes and hexagonal morphology (C). Other differentially shaped membrane structures (✱) also labelled with anti-p54 antibodies are shown in (A). Arrows indicate viral membranes. Bars, 100 nm.

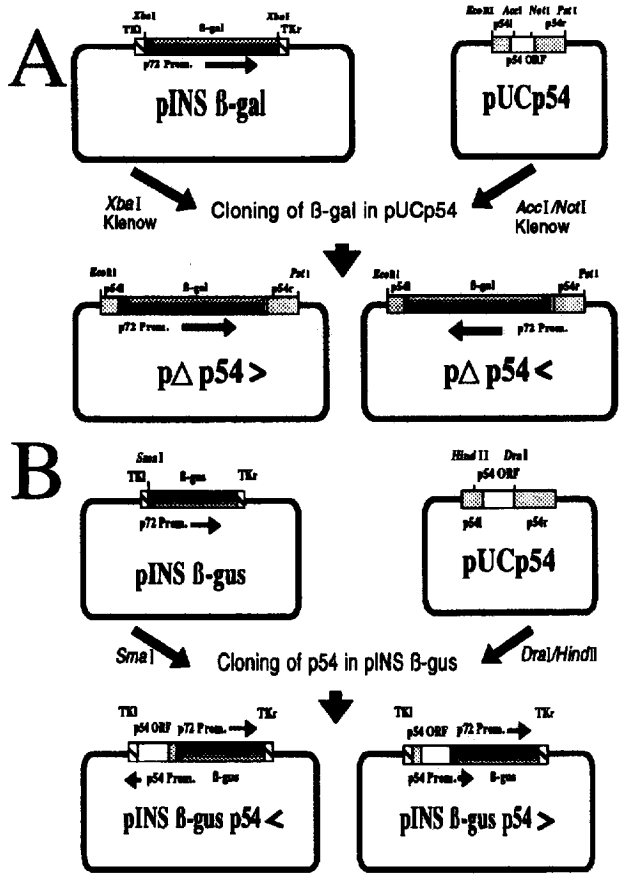

Fig. 2. Schematic representation of the constructs designed to analyze the essentiality of p54 gene. (A) Strategy for the construction and structure of the plasmids pΔp54⟩ and pΔp54⟨ used for disruption of p54 gene by insertion of β-gal by homologous recombination. Both plasmids are based on pUC19 and differ in the transcriptional direction of the marker gene. (B) Strategy for the construction and structure of the plasmids pINS β-gus p54⟩ and pINS β-gus p54⟨ used for the introduction of a second copy of p54 gene in the TK locus of the ASFV genome by homologous recombination. Both plasmids differ in the transcriptional direction of p54.

GAATT CACGTATTAAAATACTCG GCCGC and 5′-GGA CACGTCTGCAGAAAACATATC. This fragment was inserted into pUC19 and the plasmid construct was referred to as pUCp54. On the other hand, the β-galactosidase (β-gal) gene under the control of the virus promoter p72 was obtained by digestion with *Xba*I from plasmid pINS β-gal (Rodríguez et al., 1992) and treated with Klenow. Then, plasmid pUCp54 was digested with *Acc*I and *Not*I to disrupt p54 ORF and treated with Klenow to blunt the ends generated by restriction enzymes. Finally, the 3498-bp fragment containing the β-gal and p72 promoter purified by electroelution, was ligated with the resulting pUCp54 after *Acc*I/*Not*I digestion to generate the plasmids pΔp54⟩ and pΔp54⟨, whose only difference is the orientation of the β-gal gene (Fig. 2A). These vectors were constructed to facilitate the replacement, by recombination, of a genomic DNA fragment of 322 bp from the p54 gene with the reporter gene β-gal fused to the ASFV promoter p72. This would disrupt the p54 ORF and eliminate most of the sequence of the gene from the viral genome, and as a result of that, of the encoded protein (from Tyr-32 to Ala-139).

Attempts to inactivate the p54 gene from ASFV by targeted insertion of β-gal using the pΔp54 plasmids were unsuccessful. Several ASFV plaques expressing β-gal, obtained after recombination with plasmid pΔp54⟨, were detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and picked. However, none of these clones maintained the β-gal expression in sequential rounds after plaque purification, suggesting that those viruses were generated as a result of an unstable single crossover event.

The failure to inactivate the gene by β-gal insertion could be explained if the gene is essential for virus replication. However, an alternative explanation is the inability of the transfected plasmid to undergo homologous recombination with enough efficiency under our experimental conditions. To rule out this latter possibility, we constructed a virus recombinant containing an additional p54 gene into the thymidine kinase (TK) locus, and checked whether we could then delete the normal p54 gene. For this purpose we generated a new plasmid construct designed to incorporate the second copy of the p54 gene. A 760 bp *Hin*dII-*Dra*I fragment containing the complete p54 ORF and 200 bp upstream of the gene, containing the p54 promoter, was obtained by electroelution from the above described plasmid pUCp54. This fragment digested with *Sma*I was cloned into a plasmid named pINS β-gus (García et al., 1995; Gómez-Puertas et al., 1995), which contained the β-glucuronidase (β-gus) marker gene, under the control of ASFV p72 promoter, flanked by the sequences of the ASFV TK gene. This cloning resulted in two plasmids pINS β-gus p54⟩ and pINS β-gus p54⟨ (Fig. 2B), whose only difference is the orientation of the p54 gene. Homologous recombination between these plasmids and the virus resulted in the generation of stable recombinants with only plasmid pINS β-gus p54⟨. This recombinant virus contains an extra copy of the p54 gene in the TK locus in the same direction as the original p54 gene present in *Eco*RI E fragment of the virus, and in the opposite orientation to the β-gus gene. ASFV plaques in Vero cells expressing β-gus were detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc), and the viral mutant, vΔ1 β-gus (Fig. 3A), was isolated and amplified.

The Δ1 β-gus virus mutant obtained was used in new homologous recombination experiments with plasmids pΔp54⟩ and pΔp54⟨. In this virus, we were able to delete the original p54 gene, indicating that only when a second copy of this gene was incorporated into the viral genome it was possible to obtain deletion mutants. Therefore, the obtained results strongly suggest that this gene is essential for virus viability. The double recombinant virus, vΔ2 β-gus β-gal (Fig. 3B), expressed the marker genes β-gus and β-gal (Fig. 3C1). Interestingly, the double recombinant virus progeny expressing the second marker β-gal was only obtained using the plasmid pΔp54⟩. This mutant virus transcribes the β-gal gene in the same direction that p54 in *Eco*RI E genomic fragment.

DNA from the parental virus, Ba71V, and the recombinant viruses, vΔ1 β-gus and vΔ2 β-gus β-gal, was purified from infected cultures, digested with the restriction enzyme *Eco*RI, and then subjected to agarose gel electrophoresis as described (Blasco et al., 1989b). Southern blot analysis using a ^{32}P-labelled probe, consisting of 322 bp of p54 ORF obtained by digestion of pUCp54 with *Acc*I and *Not*I (Fig. 2A), and purified from agarose gel before labelling, demonstrated that the genomic structures of the recombinant viruses were as predicted. The *Acc*I-*Not*I probe hybridized to *Eco*RI E fragment of 8.8 kb in parental virus, with two fragments in vΔ1 β-gus corresponding to *Eco*RI fragments E and K, the last one with the molecular weight altered by the insertion of β-gus-p54 construction in TK locus (from 4.8–7.5 kb), and only with the modified K fragment in vΔ2 β-gus β-gal because of the deletion of the p54 original copy in E genomic fragment (Fig. 3C2). The recombinant virus vΔ2 β-gus β-gal showed colour reaction when it was incubated in the presence of X-gluc or X-gal, the β-gus and β-gal substrate respectively (Fig. 3C1).

The production of recombinant viruses expressing β-gus and a second copy of p54, was possible only when we used the plasmid pINS β-gus p54⟨, in which both genes have opposite orientation. This phenomenon could be explained by the ab-

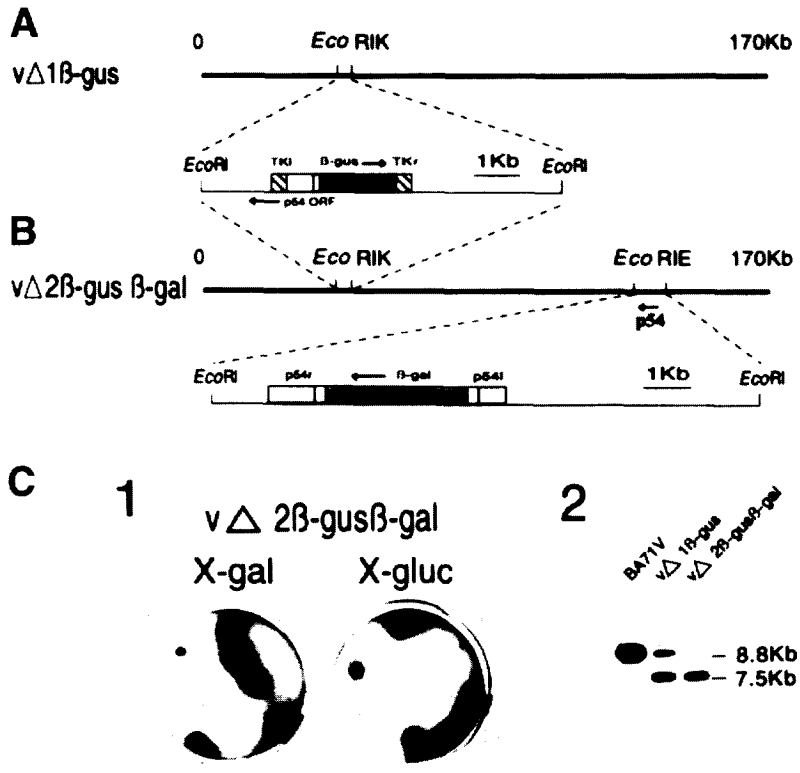

Fig. 3. Predicted genomic structure of the ASF virus recombinants vΔ1β-gus (A) and vΔ2β-gus β-gal (B). Homologous recombination between the virus genome and the plasmid vectors pINS β-gus p54$\langle$ (A) and pΔp54$\rangle$ (B) should lead the insertion of the β-gus and β-gal genes respectively, fused to the virus promoter p73 into two different *Eco*RI genomic fragments. First recombination introduced an additional copy of the p54 gene in the TK locus jointly the β-gus marker gene. Second recombination disrupted the p54 gene by insertion of β-gal marker gene. The resulting virus after the two mentioned recombinations express the two marker genes as demonstrated by staining of virus plaques with X-gal and X-gluc (C1). Genomic analysis of parental and recombinant ASF viruses by Southern blot using a DNA probe corresponding to 322 bp of p54 ORF demonstrated the presence of the different number of copies of p54 gene in the analyzed viruses (C2).

sence of specific transcription termination signals in the p54 gene. Certain orientation of p54 and β-gus could produce interferences in a normal transcription process of the β-gus gene when the virus incorporates the construction by homologous recombination. Surprisingly, the obtention of the mutant virus vΔ2β-gus β-gal, in which the original p54 copy was deleted, was possible only when the homologous recombination was carried out with pΔp54$\rangle$. This virus contains the β-gal gene in the same orientation of p54 in *Eco*RI E genomic fragment.

The second copy of p54 was incorporated in TK locus under the control of p54 promoter, localized 200 bp upstream of the p54 ORF. The transcription of the gene was efficient and protein p54 was detected in infected cells by Western blot (data not shown). Further characterization of this promoter would be useful to express other genes at late times during infection.

Although p54 can not be easily detected on the virions surrounded by the two envelopes when sections of infected cells were analyzed, biochemical data indicate that the extracellular viral particles do contain the antigen (Rodriguez et al., 1994). Therefore it is possible that the epitope is masked when the virus acquires new envelopes due to conformational changes or more simply, access of the antibody to the antigen may be reduced. A similar phenomenon has been de-

scribed with the vaccinia protein p65 (Sodeik et al., 1994). Further investigations to understand the specific role of p54 gene during the virus morphogenesis and replicative cycle are currently under way.

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