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# Characterization and Molecular Basis of Heterogeneity of the African Swine Fever Virus Envelope Protein p54

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**It has been reported that the propagation of African swine fever virus (ASFV) in cell culture generates viral subpopulations differing in protein p54 (C. Alcaraz, A. Brun, F. Ruiz-Gonzalvo, and J. M. Escribano, Virus Res. 23:173–182, 1992). A recombinant bacteriophage expressing a 328-bp fragment of the p54 gene was selected in a lambda phage expression library of ASFV genomic fragments by immunoscreening with antibodies against p54 protein. The sequence of this recombinant phage allowed the location of the p54 gene in the *Eco*RI E fragment of the ASFV genome. Nucleotide sequence obtained from this fragment revealed an open reading frame encoding a protein of 183 amino acids with a calculated molecular weight of 19,861. This protein contains a transmembrane domain and a Gly-Gly-X motif, a recognition sequence for protein processing of several ASFV structural proteins. In addition, two direct tandem repetitions were also found within this open reading frame. Further characterization of the transcription and gene product revealed that the p54 gene is translated from a late mRNA and the protein is incorporated to the external membrane of the virus particle. A comparison of the nucleotide sequence of the p54 gene carried by two virulent ASFV strains (E70 and E75) with that obtained from virus Ba71V showed 100% similarity. However, when p54 genes from viral clones generated by cell culture passage and coding for p54 proteins with different electrophoretic mobility were sequenced, they showed changes in the number of copies of a 12-nucleotide sequence repeat. These changes produce alterations in the number of copies of the amino acid sequence Pro-Ala-Ala-Ala present in p54, resulting in stepwise modifications in the molecular weight of the protein. These duplications and deletions of a tandem repeat sequence array within a protein coding region constitute a novel mechanism of genetic diversification in ASFV.**

African swine fever virus (ASFV) is the causative agent of an important disease of swine, African swine fever. In addition to domestic pigs, ASFV also infects other members of the Suidae family and soft ticks of the *Ornithodoros* genus (55). African swine fever shows a wide variety of clinical forms, ranging from mild to peracute forms of the disease (22). The lack of an effective vaccine makes ASFV, for many countries worldwide, a major animal health problem.

The virus particles consist of large icosahedral capsids, similar to those of iridoviruses (29), wrapped by a lipoprotein membrane. The virus genome, a single molecule of double-stranded DNA of about 170 kb with covalently linked ends, has an estimated coding capacity of more than 100 polypeptides (6, 41), of which at least 34 are thought to correspond to structural proteins (14).

In this report, we describe the molecular cloning and expression analysis of the gene coding for p54, a very immunogenic ASFV protein. p54 is one of the first ASFV-encoded polypeptides inducing specific antibodies after inoculation of pigs with attenuated virus strains (32). We have recently shown that, during the process of adaptation to tissue culture, changes in the apparent molecular mass of the ASFV-specified protein p54 are produced (6). Interestingly, a direct correlation between the number of passages in tissue culture and both the extent of these changes and the number of the generated p54 variants was found (6). Thus, we were keen to study the molecular basis for this heterogeneity.

Numerous reports have described antigenic and genetic heterogeneity as well as differences in biological properties among different ASFV isolates or clones derived from the same parental strain. This heterogeneity seems to be generated, both in nature (3, 12, 31, 48, 54) and in vitro, during cell culture passage (6, 12, 21, 42, 50, 54). Two mechanisms for the diversification of the ASFV genome have been described: (i) changes in the lengths of unique and repeated sequences located near the genome ends (3, 13, 17), and (ii) variations in both the numbers and the nucleotide sequences of tandem repeats found at some intergenic regions (10, 19). These mechanisms of diversification imply the occurrence, during the virus replicative cycle, of homologous and nonhomologous recombination events as well as separate processes of DNA rearrangement. In addition, another mechanism by which variation in the numbers of direct repeats might have arisen is polymerase stuttering or jumping as described elsewhere (4, 25). The biological consequences of the ASFV diversity are, as yet, poorly understood.

The results presented here demonstrate, for the first time, that ASFV genetic heterogeneity can be the result of alterations in the number of units of tandemly repeated sequences found within the coding sequences of virus genes.

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## MATERIALS AND METHODS

**Cells and viruses.** Vero and monkey stable (MS) cell lines were grown in Dulbecco's modified Eagle's medium containing 10 and 5%, respectively, fetal bovine serum. The virulent ASFV isolates E70, E75, 608, and 646 were isolated between 1968 and 1977 from natural outbreaks taking place in different areas of Spain. None of these virus isolates were passaged for more than 8 times in buffy coat cultures before nucleotide sequencing or Western immunoblot analyses.

Vero and MS cell lines were used for the adaptation of wild-type viruses. The adapted viruses used for p54 analysis were E70 VR90, E75 VR85, E75 VR3, 608 VR79, 646 VR85, E75 MS23, and E70 adapted to MS cells in passages 15, 16, 44, 81, 97, 102, 107, and 113, respectively. Virus clones generated from E70 MS81 virus were purified by limit dilution.

Extracellular BA71V virus particles were purified by Percoll equilibrium centrifugation as described elsewhere (14) for analysis of the virus structural proteins. Viral cores were obtained by treatment of virus particles with the nonionic detergent Nonidet P-40 (49).

Virus-infected cell extracts (20) at 18 h postinfection were obtained in the presence of cytosine arabinoside (40 μg/ml) for early protein synthesis characterization or without inhibitor of DNA synthesis for late proteins.

Protein analyses of purified virus and cell extracts were performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting techniques as previously described (7).

**Screening of a lambda phage ASFV genomic library.** A library prepared from randomly generated fragments of the ASFV virulent strain E75 genome within the lambda phage expression vector λZapII (1) was immunoscreened by use of p54-specific antibodies. The antibodies were affinity purified, by a modification of the Western blot technique (5, 30, 45), from serum obtained from NIH miniature pigs (38) ($SLA^{aa}$) inoculated orally with the attenuated ASFV E75 CV1-4 strain (5). After the immunoscreening, the nucleotide sequence corresponding to the insert of a positive phage clone was determined by the dideoxynucleotide chain terminator method (40), with M13 sequencing primers.

**DNA sequencing and computer analysis.** Amplification of the p54 gene from different virus isolates was carried out by PCR with AmpliTaq DNA polymerase (Perkin-Elmer Cetus) with the following primers: (i) 5′-GCGCGCGAATTCCTT GAGAATACTTGGAAAG (5′ primer), which includes the recognition sequence for the *Eco*RI restriction enzyme, and (ii) 5′-GCGCGCGGATCCGTAGCTAATAAGCTCTGAGC (3′ primer), which contains the recognition sequence for the *Bam*HI restriction enzyme. The DNA templates used for the amplification reactions were obtained either from peripheral blood of infected pigs (12), for the wild-type viruses, or from supernatants of infected cell cultures (13), for viruses adapted to grow in tissue culture. The different PCR products were digested with *Eco*RI and *Bam*HI and cloned into pUC 19, previously digested with the same enzymes according to standard procedures (39). The cloned p54 genes were sequenced by the dideoxynucleotide chain terminator method with M13-specific primers and the p54 primers 5′-CTTGAGAATACT TGGAAAGTTGGTCC, 5′-ATGGATTCTGAATTTTTTC AACCGG, 5′-GCTGCCGCTATTGAGGAGG, and 5′-CAA CCCAGTTACGGACAGAC (5′ primers) and 5′-ATGAC TAAGACCACGATAGC, 5′-GCTGTAGTCGCTCCAGCC GG, 5′-GCGTATAGGTGTTTCTTTGTCG, and 5′-CGTA GCTAATAAGCTCTGAGC (3′ primers).

DNA and protein sequences were analyzed by using the PC/Gene software package (IntelliGenetics Inc.) and the programs of the Genetics Computer Group, University of Wisconsin (18).

**Preparation and analysis of RNA.** Total RNA was obtained from Vero cell cultures infected with 25 PFU of the BA71V strain of ASFV per cell as described elsewhere (39). Briefly, after removal of the medium, cells were scraped, resuspended in lysis buffer (4 M guanidinium thiocyanate, 0.1 M Tris-HCl [pH 7.5], 1% β-mercaptoethanol), layered on top of a 3-ml cushion of 5.7 M cesium chloride containing 10 mM EDTA, and spun at 300,000 × *g* for 18 h. The RNAs were resuspended in $H_2O$ pretreated with diethyl pyrocarbonate (DEPC; 0.2%) containing 1% SDS and ethanol precipitated twice. The RNAs were finally resuspended in DEPC-treated $H_2O$ at a concentration of 2 mg/ml. ASFV immediate-early and early RNA samples from infected cells maintained in the presence of early RNA samples were obtained at 8 h postinfection from infected cells maintained in the presence of 100 μg of cycloheximide per ml or 40 μg of cytosine arabinoside per ml, respectively. Late RNA was obtained at 18 h postinfection, in the absence of inhibitors. The RNA hybridization and primer extension analyses of the p54 gene were carried out as previously described (8), with the oligonucleotide primer 5′-TGTGGAGAAGAAG CTTGGTGGGGTGACTGG.

**Construction of p54 expression plasmids and generation of a p54-specific antiserum.** A recombinant plasmid, pEXE1, containing the p54 gene from the E70 ASFV strain fused to the N-terminal part of MS2 polymerase under the control of the inducible λPL promoter, was constructed by cloning the PCR-amplified ASFV gene into the pEX 34 expression vector (47). The amplification was carried out with the following primers: (i) 5′-GCGCGCGAATTCATGGATTCTGAATTTTTT CAACC, which contains the initiation codon of the p54 open reading frame (ORF), and (ii) 5′-GCGCGCGGATCCGTAG CTAATAAGCTCTGAGC. The PCR products were digested with *Eco*RI and *Bam*HI and then cloned into the polylinker region of pEX 34. The expressed p54-MS2 fusion protein was partially purified by sequential treatment with different concentrations of urea (47). The antigenic specificity of the fusion protein was confirmed by Western blotting, with the affinity-purified p54-specific antibodies described above, and the protein was used to raise a high-titer pig antiserum. For this, a 225-μg dose of the recombinant protein was inoculated intramuscularly in the first inoculation in the presence of complete Freund's adjuvant. A second inoculation was done with the protein in the presence of incomplete Freund's adjuvant. Finally, a third dose of recombinant protein was inoculated 2 weeks later and the pig was bled. The antiserum obtained showed titers of antibodies higher than 1:2,000 in Western blot. This antiserum was used in all immunofluorescence and Western blot analyses.

**Immunofluorescence analysis.** ASFV-infected MS cells at 12 hours postinfection were washed with staining buffer (10 mM EDTA in PBS, pH 7.2) at 4°C and incubated with the monospecific anti-p54 serum at a 1:100 dilution for 30 min at 4°C. Then, cells were washed twice with staining buffer and incubated with a second fluorescein isothiocyanate rabbit anti-swine immunoglobulin G at a 1:30 dilution for 30 min at 4°C. After two more washes with staining buffer, cells were observed by microscope.

## RESULTS

**Molecular cloning and nucleotide sequence of the p54 gene.** The strategy used for the molecular cloning of the p54 gene was based on the immunoscreening, with affinity-purified p54

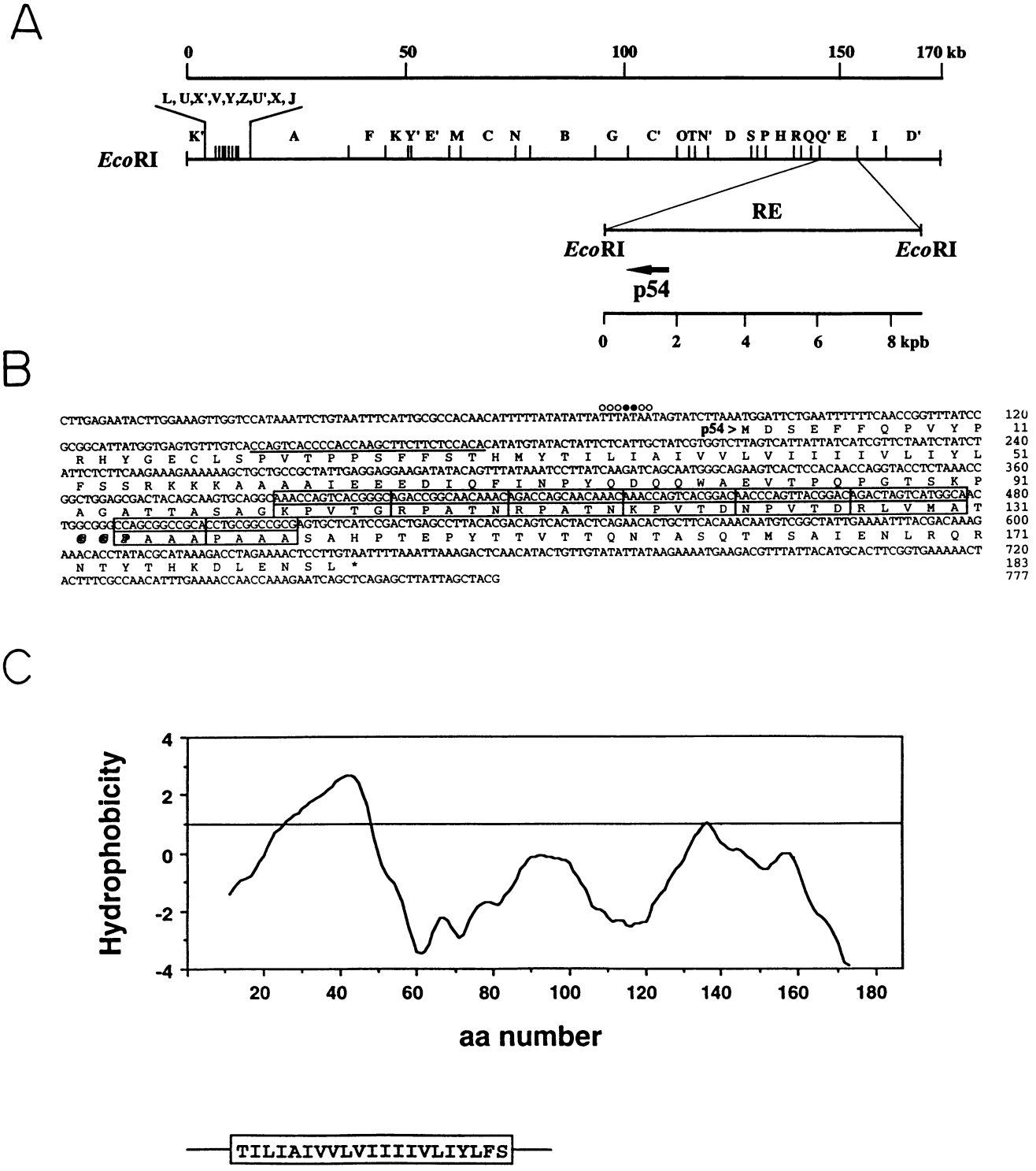


FIG. 1. Sequence of the gene encoding protein p54. (A) Schematic representation of the mapping of the gene in the ASFV genome within the *Eco*RI E fragment. The arrow indicates the transcriptional direction. (B) Nucleotide sequence and deduced amino acid sequence of the p54 gene and flanking regions. Oligonucleotides complementary to the underlined sequences were used as hybridization probes in the primer extension and in the Northern (RNA) blot analyses. Outlined lettering indicates the position of the consensus proteolytic sequence Gly-Gly-X. The filled and empty circles indicates majoritary and minoritary potential transcriptional initiation sites detected by primer extension. Boxed sequences represent the two direct repetitions detected in the p54 gene. (C) Hydrophobicity profile of the p54 protein. The boxed sequence below represents the transmembrane domain.

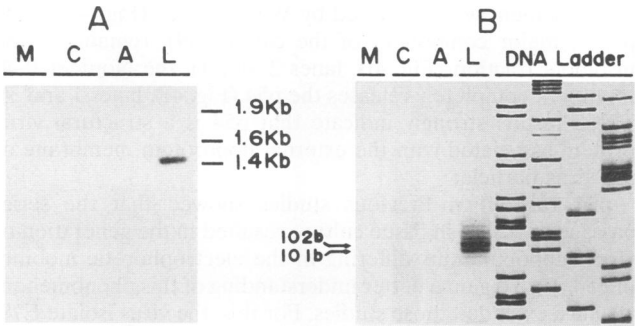


FIG. 2. Transcriptional analysis of the p54 gene. Autoradiograms of RNA hybridization (A) and primer extension analysis (B) of four types of RNA, mock infected (lanes M), ASFV virus-induced immediate-early (lanes C), early (lanes A), and late (lanes L), are shown. In panel A, the position of the most prominent late transcript is shown. The samples in panel B were electrophoresed alongside an irrelevant DNA-sequencing reaction (DNA ladder) used as a size marker. Numbers at the left correspond to the sizes (in bases [b]) of the relevant DNA fragments.

specific antibodies, of a lambda phage expression library generated from the genome of the E75 strain of ASFV (1). After the screening, a recombinant phage expressing a β-galactosidase fusion protein recognized by the p54-specific antibodies was identified and isolated. This phage contains nucleotides 72 to 400 of the p54 ORF. The nucleotide sequence of this insert was determined and used to locate the p54 gene in a data bank containing the complete nucleotide sequence of the BA71V ASFV strain (56). The results of this search revealed that the p54 ORF maps within the *Eco*RI E fragment of the virus genome and is transcribed leftwards (Fig. 1A). This ORF encodes a protein with a predicted isoelectric point of 6.5 and a calculated molecular mass of 19,861 Da (Fig. 1B). The predicted protein size does not correspond to the observed mobility of viral p54 in SDS-PAGE. Anomalous electrophoretic mobility was also observed in the p54 fusion protein expressed in *Escherichia coli*, showing an apparent molecular mass of 35 kDa instead the expected 29 kDa (data not shown). The hydropathy profile of p54 (Fig. 1C) reveals the presence of a very hydrophobic stretch, formed by 21 amino acid residues, within the N-terminal region (residues 33 to 53) of the protein, which most likely represents a transmembrane domain (18).

Two types of imperfect direct repeats were found within the coding sequence of the p54 gene (Fig. 1B). The first one is formed by six tandem repeats of a 15-nucleotide-long sequence, while the second one consists of a stretch of 12 nucleotides that is found twice.

**Transcriptional analysis of the p54 gene.** The transcriptional features of the p54 gene were analyzed by RNA hybridization and primer extension. For these experiments, total RNA from mock-infected Vero cells (uninfected RNA) or virus-infected cultures maintained in the presence of either cycloheximide (immediate early RNA) or 1-β-D-arabinofuranosylcytosine (early RNA) or in the absence of inhibitors (late RNA) were used. Both the RNA hybridization and primer extension were performed with a $^{32}$P-end-labeled oligonucleotide complementary to the region spanning nucleotides 58 to 87 of the coding strand of the p54 gene (see Fig. 1B). The results of the RNA hybridization (Fig. 2A) demonstrate the existence of a virus-induced late mRNA species of approximately 1.45 kb specifically recognized by the oligonucleotide probe. Similarly, the formation of primer-extended products was detected only in the sample corresponding to virus-induced late RNA, in which two major DNA products of 101 to 102 nucleotides were synthesized (Fig. 2B). This result demonstrates the presence of a late transcriptional initiation site 14 to 15 nucleotides upstream of the initiation codon of the p54 ORF (Fig. 1B). These results indicate that the p54 gene is transcribed only during the late phase of the infectious cycle. In agreement with this, the synthesis of p54 is detectable only after virus DNA replication (data not shown).

**Localization of p54 in infected cells and purified ASFV virions.** The distribution of p54 was analyzed by immunofluorescence of ASFV-infected MS cells by use of the specific antiserum against the p54-MS2 fusion protein. As shown in Fig. 3, most of the immunofluorescence corresponding to p54 accumulates to form large and bright spots in the cytoplasm of the infected cells, close to the nucleus, corresponding to the viral factories where viral morphogenesis takes place (43, 44). Interestingly, no specific staining associated with the plasma membrane of the infected cells was detected.

Previous experiments, carried out with semipurified ASFV particles, suggested that p54 might be a structural virus

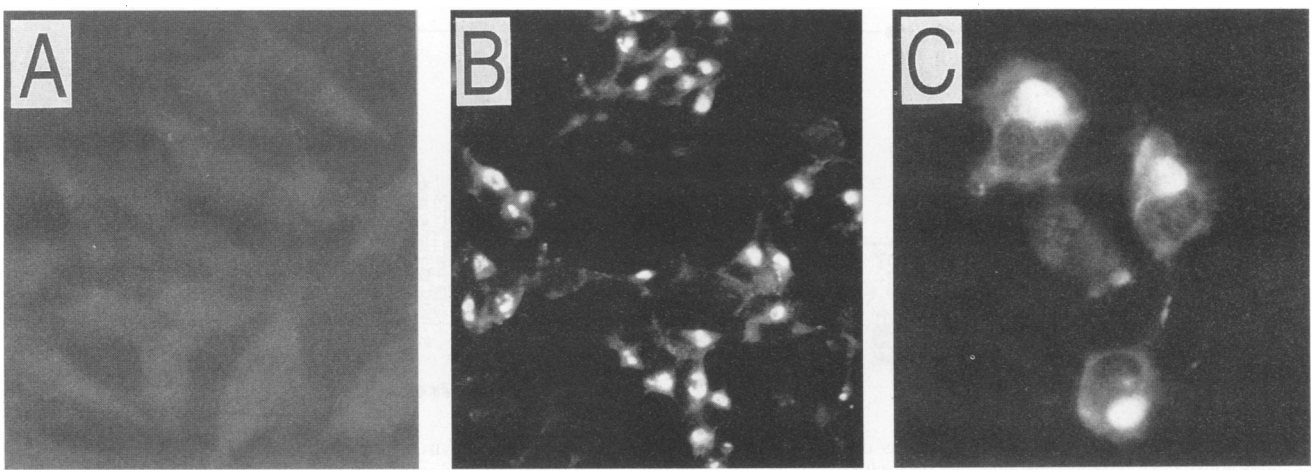


FIG. 3. Distribution of p54 protein in ASFV-infected cells. Antibody immunofluorescence of uninfected (A) and ASFV-infected cells at 12 h after infection and at two different magnifications (B and C), with a pig antiserum against the p54 recombinant protein expressed in *E. coli*.

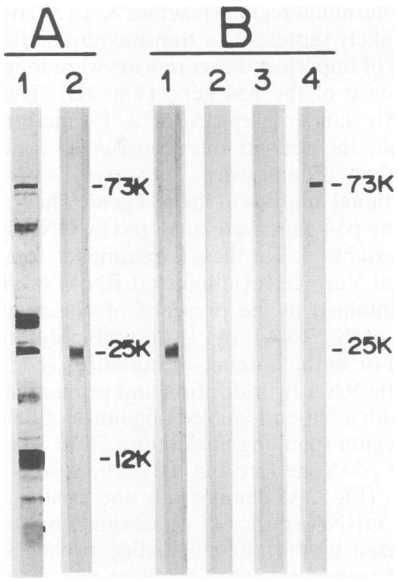


FIG. 4. Identification of p54 protein as incorporated to the virus particles by Western blot. (A) Immunoreaction of a hyperimmune serum (lane 1) and a serum against p54 (lane 2) with ASFV proteins. (B) Nonidet P-40-released (lanes 1 and 2) and viral core proteins (lanes 3 and 4) immunoreacted with an antiserum against p54 protein (lanes 1 and 3) or with the monoclonal antibody 1G8G7 directed against protein p73 (lanes 2 and 4). Molecular masses (in kilodaltons) are indicated at the right of each panel.

protein. To further study that possibility, the presence of p54 within highly purified ASFV particles (14) was analyzed by Western blot. As shown in Fig. 4, ASFV purified virions contain significant amounts of p54 (Fig. 4A, lane 2). In an attempt to determine the location of p54 within the virus particle, purified virions were treated with the nonionic detergent Nonidet P-40 and then subjected to ultracentrifugation. Both the supernatant and the pellet fractions obtained after this treatment were analyzed by Western blot (Fig. 4). While p73, a major component of the capsid (49), remains in the insoluble fraction (Fig. 4B, lanes 2 and 4), the Nonidet P-40 treatment completely releases the p54 (Fig. 4B, lanes 1 and 3). These results strongly indicate that p54 is a structural virus protein associated with the external lipoprotein membrane of the virus particle.

**p54 variability.** Previous studies showed that the serial passage of ASFV in tissue culture resulted in the generation of virus subpopulations differing in the electrophoretic mobility of p54 (6). To gain a better understanding of this phenomenon, we have extended those studies. For this, the virus isolate E70, which had been maintained for 81 consecutive rounds of passage in MS cells (6), was passaged another 32 times in the same cell line. The alterations of the electrophoretic mobility of the different p54 subpopulations produced throughout the whole process were investigated by Western blot analysis. The five different viral subpopulations differing in the molecular weight of p54 detected in passage 81 were maintained until passage 113 (Fig. 5A). However, the relative proportion corresponding to each particular virus subpopulation, as quantified by densitometric analysis of the Western blots, significantly varies with the passage number (Fig. 5B). Thus, while after the first 80 passages the most prominent virus subpopulations expressed two forms of p54 with electrophoretic mobilities corresponding to molecular masses of 27 and 28 kDa, from passages 81 to 113 the prevalence corresponds to a subpopulation expressing a smaller form of the protein (26 kDa).

To explore whether the generation of changes affecting the electrophoretic mobility of p54 might be dependent upon the cell line or the ASFV strain used for the experiments, the mobility of the p54 encoded by four different ASFV strains adapted either to MS or Vero cells and subjected to different numbers of replication rounds in tissue culture was analyzed. Each of the tissue-adapted ASFV strains used in this study showed a difference in the electrophoretic mobility of its p54 with respect to that expressed by its corresponding nonadapted parental virus strain (Fig. 6). In contrast, regardless of their origin, each of the wild-type viruses analyzed in our study

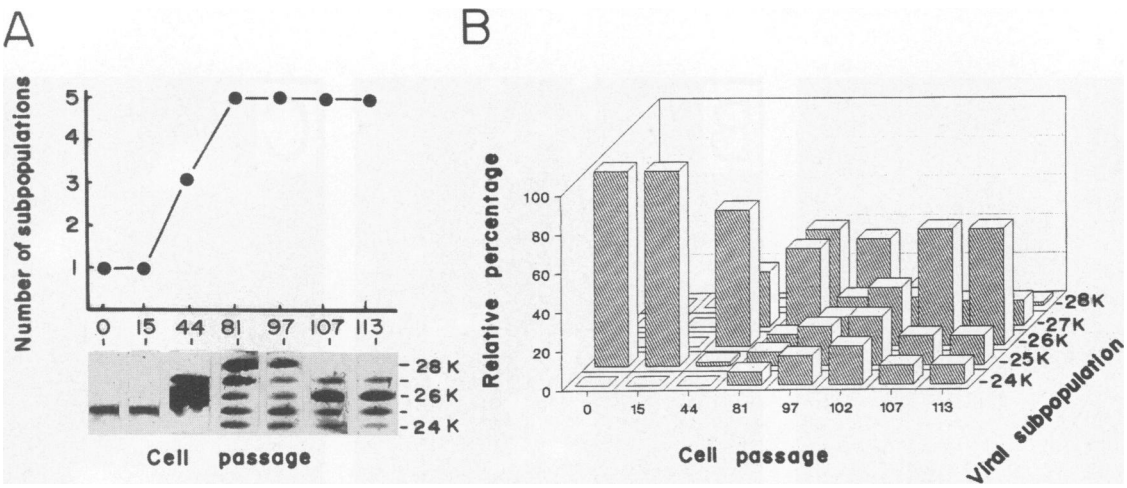


FIG. 5. Generation of virus subpopulations during cell culture passage. (A) Number of virus subpopulations generated along cell culture passages (0 to 113), detected by Western blot with a pig antiserum against protein p54. (B) Quantitation of the relative percentage of each viral subpopulation coding for different forms of p54 as deduced by densitograms of the Western blots shown in panel A. Molecular masses (in kilodaltons) are shown at the lower right of each panel.

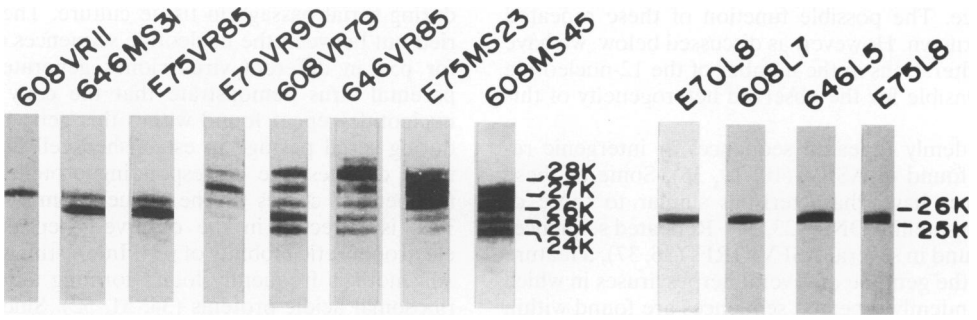


FIG. 6. Diversity of p54 protein forms, detected by Western blot with a pig antiserum against this protein, coded by four virus strains (E70, E75, 608, and 646) adapted to growth in monkey stable (MS) and Vero (VR) cells or grown only in pig macrophages (L). Molecular masses (in kilodaltons) are shown at the right of each panel.

expresses a p54 protein with exactly the same electrophoretic mobility (25 kDa) (Fig. 6).

**p54 heterogeneity is the result of mutations generated during serial passage.** To characterize the genetic changes affecting the electrophoretic mobility of p54, the p54 genes of several virus strains were isolated by PCR and cloned into the plasmid vector pUC 19, and their corresponding nucleotide sequences were determined by the dideoxynucleotide termination method by use of specific primers. Initially, this analysis was carried out with the following viruses: (i) wild-type E75 and E70 viruses, (ii) E70 MS15, a derivative of E70 that had been passaged 15 times in MS cells, which expresses a p54 with an electrophoretic mobility identical to that of the parental virus E70 (Fig. 5), and (iii) virus clones 2, 15, and 29, obtained by limiting dilution from a stock of E70 generated after 81 consecutive passages in MS cells. The results of this analysis showed that the p54 genes of E70 and E75 wild-type viruses and E70 MS15 virus are identical to their counterpart in BA71V. However, sequence differences among the p54 genes from virus clones 2, 15, and 29 were detected. These differences consist in changes in the number of copies of an imperfect direct repeat formed by 12 nucleotides that is found twice within the p54 genes of BA71V, E75, E70, and E70 MS15 (Fig. 7). Clones 15 and 2 contain three and four copies, respectively, of this sequence repeat, while clone 29 contains a single copy (Fig. 7). This repeated sequence encodes the tetrapeptide Pro-Ala-Ala-Ala. Thus, its seems clear that the stepwise changes observed in the electrophoretic mobility of p54 are caused by the alteration in the number of Pro-Ala-Ala-Ala blocks found within the protein.

## DISCUSSION

We have determined the physical map and the nucleotide sequence of the gene encoding p54, a very immunogenic ASFV protein. One of the most interesting features of this gene consists in the presence, within its coding region, of two groups of tandemly repeated sequences. The first group is formed by six repeats of a sequence formed by 15 nucleotides, while the second one consists of two imperfect repeats of a 12-nucleo-

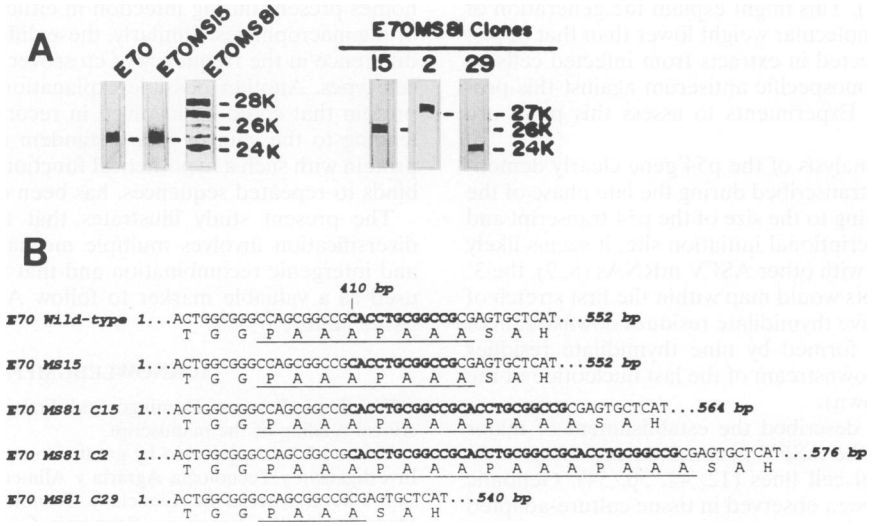


FIG. 7. (A) Generation of viral subpopulations detected by Western blot with a pig serum against protein p54 in the virus E70 after passage in the MS cell line. Three virus clones (15, 2 and 29) coding for one form of p54 and segregated by limit dilution from passage 81 are also shown. (B) Modifications in p54 gene sequences obtained from E70 wild-type virus, from the same virus after 15 passages in MS cells (E70 MS15), and from viral clones obtained from MS passage 81 and shown in panel A (E70 MS81 C15, C2, and C29). The nucleotide sequences shown in boldface type correspond with the copies of the variable repetitive sequence in the p54 genes. The underlined sequences correspond with the amino acid repetitive motifs in p54 proteins.

tide-long sequence. The possible function of these repeated sequences is not known. However, as discussed below, we have established that alterations in the number of the 12-nucleotide repeats are responsible for the observed heterogeneity of this protein (6).

Previously, tandemly repeated sequences at intergenic regions have been found in ASFV (10, 19, 36). Some of these repeated sequences have characteristics similar to those of chromosomal minisatellite DNA (23, 24). Repeated sequences have also been found in several ASFV ORFs (36, 37), a feature also described in the genome of several herpesviruses in which short blocks of tandemly repeated sequences are found within the coding regions of certain genes (16, 28, 35, 57). In addition, short direct repeats within intergenic regions and coding regions that vary between different strains of vaccinia virus or between vaccinia virus and variola have been described (2, 46). However, duplicated sequences introduced into the vaccinia virus genome were unstable (11).

The hydropathy profile of p54 contains the elements found in type II transmembrane proteins (53): (i) a hydrophilic N-terminal domain formed by 32 residues, (ii) a very hydrophobic stretch of 21 amino acid residues near the N terminus of the polypeptide (residues 33 to 53), immediately followed by three positively charged residues, and (iii) a hydrophilic C terminus formed by 130 amino acid residues. According to this, it seems likely that p54 is a transmembrane protein with a short N-terminal domain exposed in the external surface of the membrane and a large C-terminal tail facing the internal region. Western blot analysis with highly purified virus particles has demonstrated that p54 is a structural virus protein that associates with the external lipoprotein membrane of the virion. Interestingly, during the virus replicative cycle, p54 accumulates within perinuclear areas of the infected cell, suggesting that ASFV particles acquire their lipoprotein membrane within the virus factories. Thus, p54-specific antibodies could become a very helpful tool to analyze the last stages of ASFV morphogenesis.

The presence, within the predicted cytoplasmic tail of p54, of the sequence motif Gly-Gly-X, a consensus sequence for the proteolytic processing of at least three structural ASFV proteins, is noteworthy (26). This might explain the generation of two protein species of molecular weight lower than that of p54 that are frequently detected in extracts from infected cells by Western blot with a monospecific antiserum against this protein (data not shown). Experiments to assess this point are currently under way.

The transcriptional analysis of the p54 gene clearly demonstrated that this gene is transcribed during the late phase of the infectious cycle. According to the size of the p54 transcript and the position of its transcriptional initiation site, it seems likely that, as has been shown with other ASFV mRNAs (8, 9), the 3′ end of the p54 transcripts would map within the first stretch of seven or more consecutive thymidilate residues downstream of the ORF. A sequence formed by nine thymidilate residues (9T) is located 772 bp downstream of the last nucleotide of the p54 ORF (data not shown).

Several reports have described the establishment of major rearrangements within the ASFV genome during the process of adaptation to several cell lines (12, 42, 50, 54). Genomic heterogeneity has also been observed in tissue culture-adapted virus strains after serial passage. It has been shown that plaque-purified virus clones contain deletions and/or additions within regions close to both termini of the virus genome (42). In this report, we have identified a tandemly repeated sequence, located within the coding region of the structural viral protein p54, which is a determinant of ASFV diversification during serial passage in tissue culture. The comparisons carried out between the nucleotide sequences of the gene coding for p54 in different virus clones generated from the same parental virus demonstrate that the copy number of a 12-nucleotide repeat found within this gene undergoes changes during serial passage in established cell lines. As a result of these changes, the corresponding proteins contain different numbers of copies of the sequence motif Pro-Ala-Ala-Ala. This is reflected in the observed stepwise changes in the electrophoretic mobility of p54. Interestingly, the Pro-Ala-Ala-Ala motif is frequently found forming tandem repetitions in ribosomal acidic proteins (34, 51, 52). Since all virus isolates tested after a certain number of passages in cell lines showed the presence of viral subpopulations differing in the molecular weight of the p54 protein, its seems likely that the rearrangements of the 12-nucleotide repeat might be a common phenomenon during ASFV propagation in cell culture. The fact that none of the p54 subpopulations become completely dominant during propagation in cell culture indicates that these changes do not confer a major advantage for virus replication in tissue culture. This hypothesis has been corroborated by comparative titration of different virus subpopulations in a cell line (6).

In previous studies, carried out with nonvirulent viruses, we showed that changes in the electrophoretic mobility of p54 are not related either to the replication phenotype of the virus in pig macrophages or to the restoration of virulence (6). Surprisingly, although alterations affecting the genome structure and both antigenic and biological properties of ASFV have been detected during in vivo infections (21, 31), we have not detected the generation of p54 virus subpopulations in viruses isolated from pigs after several days or weeks of viremia. Therefore, changes in p54 during pig infection seem to be more restricted than during cell culture propagation since no virus subpopulations differing in p54 were detected in wild-type virus after replication in mononuclear phagocytes (6, 27, 33).

The observed discrepancy between in vivo and in vitro generation of diversity could be the result of differences in the numbers of replication cycles or copy numbers of virus genomes present during infection in either established cell lines or pig macrophages. Similarly, these differences might reflect a difference in the frequency of crossover events in the different cell types. Another possible explanation is that in cell lines a protein that could be involved in recombination mechanisms leading to the gain or loss of tandem repeats might exist. A protein with such a hypothetical function in human cells, which binds to repeated sequences, has been described (15, 24).

The present study illustrates that the process of ASFV diversification involves multiple mechanisms including intra- and intergenic recombination and that the p54 gene could be used as a valuable marker to follow ASFV diversification in tissue culture.

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