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NOTES

Comparison of the Sequence of the Gene Encoding African Swine Fever Virus Attachment Protein p12 from Field Virus Isolates and Viruses Passaged in Tissue Culture

ANA ANGULO, ELADIO VIÑUELA,* AND ANTONIO ALCAMÍ†

Centro de Biología Molecular, Facultad de Ciencias, Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain

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Comparison of the amino acid sequence of the African swine fever virus attachment protein p12 from different field virus isolates, deduced from the nucleotide sequence of the gene, revealed a high degree of conservation. No mutations were found after adaptation to Vero cells, and a polypeptide with similar characteristics was present in an IBRS2-adapted virus. The sequence of the 5′ flanking region was conserved among the isolates, whereas sequences downstream of the gene were highly variable in length and contained direct repeats in tandem that may account for the deletions found in different isolates. Protein p12 was synthesized in swine macrophages infected with all of the viruses tested.

African swine fever (ASF) virus, an enveloped deoxyvirus, is the causative agent of a disease of domestic pigs (for reviews, see references 10, 17, 33, and 34). The virus elicits in infected pigs and virus-resistant animal species inoculated with ASF virus a peculiar immune response in which no neutralizing antibodies are produced (12). The attachment of virus particles of the Vero-adapted virus strain BA71V to Vero cells and swine macrophages is mediated by specific receptors (3, 4); it has been shown recently that the structural virus protein p12 is involved in this interaction (9). Protein p12 is encoded by open reading frame (ORF) O61R, which is located in fragment *Eco*RI-O of the genome of the Vero-adapted virus strain BA71V and is predicted to encode a polypeptide of 61 amino acids (2). The protein is characterized by the presence of a putative hydrophobic transmembrane domain in the central region that might anchor the polypeptide chain in the external virus envelope and by a cysteine-rich domain in the C-terminal region that may account for the multimerization of the protein through disulfide bonds. No posttranslational modifications of the polypeptide, such as glycosylation, phosphorylation, or fatty acid acylation, have been found in virus-infected cells or purified virions (2).

Since ASF virus replication is mainly restricted to porcine monocytes and macrophages in natural infections (22, 24), it was of interest to determine the sequence of the gene in field virus isolates because mutations in the protein may have been selected during the adaptation process of BA71V to Vero cells, the strain in which the sequence of the gene was determined (2). Also of interest was a comparison of the sequence of the protein among different natural isolates to determine whether variability of this virus attachment protein, which could be a mechanism used by ASF virus to evade the host immune response, occurs. On the other hand, variation in the sequence of protein p12 might influence the pathogenicity of the virus isolate.

In this study, we have analyzed the variability of the gene encoding protein p12 by determining and comparing the sequence in 11 virus strains corresponding to 6 field virus isolates, a virus passaged 100 times in porcine macrophages, and 4 viruses adapted to grow in established cell lines. Labeling experiments with [^{35}S]methionine and immunoprecipitation with specific antibodies against protein p12 indicate that the gene is translated during the infection of swine macrophages with all of the viruses tested.

Table 1 shows the properties of the virus isolates used in this study, which were obtained from different sources (6). Natural virus isolates (BA71, LIS57, SPE51, and TEN61) and viruses adapted to grow in Vero cells (BA71V, KIR69AV, and KIR69EV) were cloned previously by limit dilution in pig macrophages and by plaque assay in cell monolayers, respectively (6, 19). BA71H is the BA71 isolate passaged approximately 100 times in pig macrophages (19). KIR69A and KIR69E are two naturally occurring variants isolated by limit dilution from the uncloned KIR69 stock (1). KIR69A was the predominant virus in the original population, and KIR69E differs in a single deletion of 16 kb in length, located at the left end of the genome, that deletes all members of the multigene family 110 (1). UGA59I was attenuated after adaptation of the virulent isolate UGA59 to IBRS2 cells (14).

The viruses were distributed in five groups. Groups I to IV were previously classified according to the positions of the *Sal*I sites in the central region of the viral genome (6). UGA59I (group V) was not included in this previous classification. Several reasons led to the choice of these virus isolates: (i) viruses are geographically and genetically diverse, which may allow more possibilities of finding mutations in the DNA sequence; (ii) they show different degrees of pathogenicity in animals; and (iii) some of them have been adapted to grow in tissue cultures originally resistant to the infection.

* Corresponding author.
† Present address: Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom.

TABLE 1. Properties of ASF virus strains

Group	Isolate[a]	Cell tropism	Pathogenicity	Reference	Size of PCR fragment[b] (bp)
I	BA71	Mϕ[c]	Attenuated	18	390
	BA71H	Mϕ	Attenuated	19	390
	BA71V	Mϕ, Vero	Attenuated	19	390
	LIS57	Mϕ	Virulent	23	390
II	SPE51	Mϕ	Virulent	20	429 (39)
III	TEN61	Mϕ	Virulent	11	405 (15)
IV	KIR69A	Mϕ	Virulent	1	418 (28)
	KIR69AV	Mϕ, Vero	Not determined	35	418 (28)
	KIR69E	Mϕ	Virulent	1	418 (28)
	KIR69EV	Mϕ, Vero	Not determined	35	418 (28)
V	UGA59I	Mϕ, IBRS2	Attenuated	14	468 (84)

[a] Virus isolates are named by a code that indicates the origin and the isolation year. H, high passage; V, Vero adapted; A and E, different clones; I, IBRS2 adapted.

[b] Determined from the DNA sequence. The increase in size with respect to BA71V is shown in parentheses.

[c] Mϕ, macrophage.

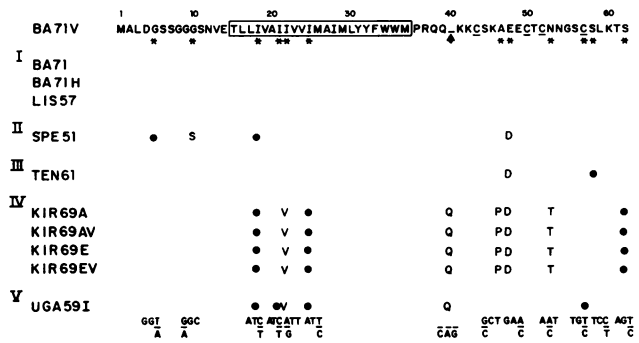

FIG. 1. Sequence of protein p12 deduced from the nucleotide sequence of the gene in different ASF virus isolates. The complete amino acid sequence of strain BA71V is shown for comparison; the boxed region corresponds to the hydrophobic segment, and the cysteine residues are underlined. The asterisks indicate the positions of the polypeptide chain that undergo mutations in other virus strains, and the arrow shows the addition of a glutamine residue. Only silent point mutations (●) and the amino acids that change with respect to the sequence of BA71V are indicated. The triplets corresponding to these positions are shown at the bottom; the nucleotide present in strain BA71V (underlined) and the mutation (below) are indicated. The base substituted was the same in the different isolates that undergo a mutation in a specific triplet.

To sequence the gene in different virus isolates, DNA fragments containing ORF O61R and the flanking regions (51 and 104 nucleotides of the 5′ and 3′ flanking sequence of BA71V, respectively, excluding the primer sequence) were amplified by the polymerase chain reaction (PCR) with specific oligonucleotides containing *Bam*HI sequences in the 5′ end. The sequences of the primers used were 5′-GCG GATCCTTGAATAAGCGTTAAC-3′ and 5′-GCGGATCCG ACATCATTATGGATGA-3′, corresponding to nucleotides 360 to 377 and 735 to 719, respectively, of the *Eco*RI-*Xba*I fragment of BA71V containing ORF O61R (2). The template used in the PCR was extracellular virus (10^5 to 10^6 hemadsorption units or PFU) that was heated at 95°C for 5 min before addition to the reaction mixture. PCR products consisted of a single band that varied in size among the virus isolates when analyzed by agarose gel electrophoresis (not shown). The lengths of the PCR fragments calculated from the DNA sequence determined are indicated in Table 1; the shortest ones were the products from group I, comprising European isolates. PCR fragments were cloned into the *Bam*HI site of plasmid pUC118 (32), and DNA sequencing was performed by the dideoxy-chain termination method (29) on single- or double-stranded templates, using a universal primer or specific oligonucleotides and Sequenase, according to standard procedures (28). The sequence was obtained for at least two independent clones for each virus isolate to confirm the fidelity of the PCR. As a control, the sequence of the gene and flanking regions in BA71V was determined by this method; the same sequence as that determined in a restriction fragment cloned from viral DNA preparations (2) was obtained. Sequences were analyzed by using the University of Wisconsin Genetics Computer Group programs (15).

Figure 1 shows the amino acid changes found in protein p12 of different virus strains in comparison with the sequence determined previously for strain BA71V. A few mutations in the sequence of the gene were observed when different groups were compared, whereas identical sequences were found when different members of the same group were analyzed (groups I and IV). These results fit well in the classification of the viruses according to the *Sal*I restriction patterns (6). The fact that some mutations found in different isolates were distributed in the same positions of the coding sequence was interesting, suggesting coevolution of viruses or the existence of mutational hot spots. The sequence of the polypeptide was highly conserved among virus isolates, since only conservative or silent point mutations were found. A conservative change of an isoleucine to a valine (groups IV and V) was the only variation found in the long stretch of hydrophobic residues present in the central region of p12. The cysteine motif of the C-terminal region was conserved in all of the viruses. The addition of a glutamine residue is interesting, giving rise to a polypeptide of 62 amino acids in the isolates belonging to groups IV and V. This addition was located in a group of hydrophilic residues (QQKK) that are predicted to constitute the highest hydrophilic segment of the polypeptide (2), and therefore it would not greatly perturb the properties of this region. Other mutations found were glycine to serine (group II), alanine to proline (group IV), glutamic acid to aspartic acid (groups II, III, and IV), and asparagine to threonine (group IV).

The conservation of the polypeptide sequence might reflect the existence of a selective pressure against mutations in the gene that may alter the basic properties of the polypeptide, suggesting that the protein encoded plays an essential role in infection. This view is supported by the fact that in several isolates (KIR69E, BA71, and BA71V), changes have been found to truncate or delete other genes that, in contrast, are dispensable for virus replication (1, 13), indicating that these viruses have had the opportunity to undergo mutations in the gene encoding protein p12. The protein did not change after 100 passages in porcine monocytes, as deduced from the sequence of BA71H. The adaptation of ASF virus to grow in Vero cells, which was studied in three viruses adapted independently (BA71V, KIR69AV, and KIR69EV), did not involve the selection of a mutated protein. If protein p12 is the only ASF virus component able to mediate attachment to the host cell, subsequent steps in the infection would be responsible for the resistance of Vero cells to natural isolates. Other factors, different from the interaction of the virus particle with the host receptor, have

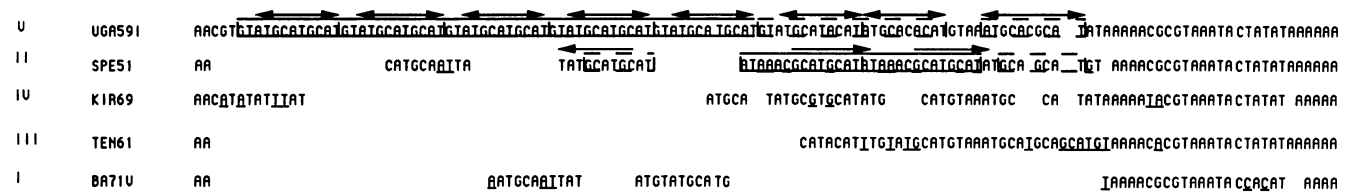

FIG. 2. Sequence of the 3′ intergenic region in different virus isolates. The alignment of the complete sequence of one member of each group is shown, since identical sequences were obtained when different members of the same group were analyzed (groups I and IV). The base substitutions with respect to UGA59I are underlined. Direct repeats are boxed, and boxes with dashed lines correspond to imperfect direct repeats. The arrows indicate the positions of the inverted repeats. The nucleotide sequence shown corresponds to nucleotides 615 to 663 of the *Eco*RI-*Xba*I fragment of BA71V (2).

been shown to play a role in determining the tropism of papovavirus, poxvirus, and retroviruses, including human immunodeficiency virus (7, 30, 31). Similarly, the selection of a protein with different characteristics does not seem to be necessary for adaptation to IBRS2 cells, although in this case the nonadapted UGA59 virus was not sequenced.

The sequence of the 51 nucleotides upstream of the initiator codon of ORF O61R was identical in all of the viruses (not shown). This sequence corresponds to an intergenic region between ORF O61R and another ORF transcribed toward the left end of the virus genome, whose initiator ATG is found 49 nucleotides upstream of ORF O61R (2). The conservation of this sequence is in agreement with the existence of transcription signals required for efficient transcription of both genes during virus infection. In accordance with this, the 5′ end of the mRNA coding for ORF O61R has been mapped within this intergenic region (25).

As in the coding region, the nucleotide sequence downstream of ORF O61R was identical among the viruses of the same group and differed when viruses belonging to different groups were compared. The 3′ flanking region downstream of ORF O61R can be divided into two segments. The first one, corresponding to an intergenic region located between nucleotides 615 and 663 of the *Eco*RI-*Xba*I fragment of BA71V, varied in length among the virus isolates sequenced (Fig. 2) and accounts for the size difference found in the PCR-amplified fragments (Table I). The second segment, located from nucleotides 664 to 718 of the *Eco*RI-*Xba*I fragment of BA71V and coding for the 3′-terminal region of another ORF transcribed leftward toward the end of the genome, was identical in length among the different viruses (not shown). This nucleotide sequence was conserved, with the exception of one or two point mutations found in each virus that corresponded to silent mutations or gave rise to conservative changes of amino acids (not shown).

Figure 2 shows that the nucleotide sequence of the 3′ intergenic region in isolate UGA59I (group V) consisted of a tandemly repeated array that contained five copies of a 12-nucleotide repeat unit, which was succeeded by three copies that differed in length or in one or two base substitutions. At the same time, 10 of the 12 nucleotides present in these repeats constituted inverted repeats. The perfect direct repeats were deleted in the other viruses. Virus SPE51 (group II) showed two perfect and two imperfect tandem direct repeats as well as inverted repeats. Both types of repeats contained part of the motifs found in UGA59I. This second type of direct repeat was partially present in viruses KIR69 (group IV) and TEN61 (group III) but was totally deleted in BA71V and the European isolates (group I), which showed the shortest sequence in this region. Prediction of secondary structures of RNA (36) indicated the formation of loops that varied in length in the different viruses, clearly influenced by the existence of inverted repeats (not shown). The biological significance of these differences is unknown. No correlation has been found between the length of this sequence and any of the properties of these viruses, such as pathogenicity or capacity to grow in different cell types. Whether these differences influence the stability or the translation efficiency of the mRNAs coding for ORF O61R or the other ORF transcribed in the other direction is presently under investigation (21). However, the fact that these direct and inverted repeats are absent in most of the isolates analyzed implies that they do not play an essential function in infection.

The existence of direct repeats might explain the genetic instability in this DNA segment. Tandemly repeated sequences of a 17-nucleotide unit, with characteristics similar to those of chromosomal minisatellite DNA, that undergo frequent DNA rearrangements have been described in an intergenic region of the ASF virus genome (16). Rodriguez et al. (26) have also described direct repeats in tandem in two intergenic regions in the *Eco*RI I fragment of the BA71V genome. However, the repeat units described here are different in both sequence and number from the other repeats found in ASF virus.

To determine whether protein p12 is synthesized during the virus replication cycle, alveolar swine macrophages (8, 27) infected with the different isolates were pulse-labeled with [^{35}S]methionine at late times of infection and immunoprecipitated with a monoclonal antibody specific for protein p12 (9) or with a rabbit antiserum raised against the protein expressed in baculovirus (5) as described elsewhere (2). Electrophoretic analysis of the immunoprecipitated proteins showed that the protein was synthesized in all of the viruses tested (not shown). Tests of the kinetics of synthesis in infected macrophages were carried out with BA71H and showed that the protein was not synthesized at early times of infection; multimers of the protein with an apparent molecular mass of 17 kDa in the absence of 2-mercaptoethanol were detected (not shown), as has been described for BA71V-infected Vero cells (2).

In summary, the nucleotide sequence of a DNA fragment containing the gene coding for protein p12 in several virus strains has shown that the 5′ flanking region is conserved in all of the virus isolates sequenced, whereas the intergenic region downstream of the gene varies among different isolates. The main properties of the polypeptide were not altered in natural isolates and viruses adapted to grow in established cell lines. The conservation of the polypeptide sequence, together with the synthesis of the protein in swine macrophages infected with all of the isolates tested, suggests

an essential role of the protein in the virus replication cycle and is in agreement with the attachment function that has been attributed to the protein (9). The results presented also indicate that the variability of protein p12, a putative virus attachment protein, is not a mechanism used by ASF virus to evade the host immune response.

Nucleotide sequence accession numbers. The DNA sequence data in this report have been submitted to the GenBank data base under accession numbers M84177, M84178, M84183, M84184, and M84185.

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