Volume 14 Number 17 1986 Nucleic Acids Research

Hairpin loop structure of African swine fever virus DNA

Antonio González, Antonio Talavera, José M.Almendral and Eladio Viñuela

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

Received 26 June 1986; Accepted 6 August 1986

ABSTRACT

The ends of African swine fever virus genome are formed by a 37 nucleotide-long hairpin loop composed, almost entirely, of incompletely paired A and T residues. The loops at each DNA end were present in two equimolar forms that, when compared in opposite polarities, were inverted and complementary (flip-flop), as in the case of poxvirus DNA. The hairpin loops of African swine fever and vaccinia virus DNAs had no homology, but both DNAs had a 16 nucleotide-long sequence, close to the hairpin loops, with an homology of about 80%. An analysis of African swine fever virus replicating DNA showed head-to-head and tail-to-tail-linked molecules that may be replicative intermediates.

INTRODUCTION

African swine fever (ASF) is an important disease of domestic pigs caused by an icosahedral cytoplasmic deoxyvirus which does not induce in the infected animals a protective immune response (reviews 1,2).

The genome of ASF virus adapted to VERO cells is a linear, double-stranded DNA of about 170 kilobase pairs (kb) (3,4), containing terminal inverted repeats (TIR) (5) and cross-links (6). These properties of ASF virus DNA as well as the presence in the virus particles of the enzymes required for the synthesis of messenger RNAs (7-10) make ASF virus similar to poxviruses (11,12).

We show in this paper that ASF virus DNA ends are hairpin loops (HPLs) composed, almost entirely, by incompletely paired A and T residues. The HPLs exist in two forms that, when compared in opposite polarities, are inverted and complementary. We also show that replicating DNA molecules isolated from ASF virus-infected VERO cells consist of polymers linked

head-to-head and tail-to-tail.

MATERIAL AND METHODS

Virus and cells.

ASF virus BA71V, adapted to grow in VERO cells (CCL81, American Type Culture Collection) was cloned by plaque purification (13). Virus stocks were obtained from VERO cells infected at a multiplicity of infection (moi) of 0.001-0.01 plaque forming units (pfu) per cell in Dulbecco's modified Eagle medium with 2% newborn calf serum. When the cytopathic effect was complete, cell debris was removed by low-speed centrifugation and the virus in the supernatant was recovered in a pellet that was resuspended in phosphate buffered saline (PBS). The virus suspension was stored in portions at -70ºC.

Viral DNA purification.

ASF virus DNA was isolated from partially purified virus particles obtained by the method of Black and Brown (14) with slight modifications. An extracellular virus suspension from about 2 x 10^9 infected VERO cells was incubated overnight at 4ºC in 0.5 ml of a mixture containing 10 mM Tris-HCl (pH 8), 10 mM EDTA, 10 mM NaCl, 0.5% Sarkosyl NL-97 and 250 µg of autodigested proteinase K (Boehringer Mannheim). After two phenol-chloroform (10:1) treatments, 0.5 ml of the aqueous phase was layered onto 13 ml of a 5-20% sucrose gradient in 10 mM Tris-HCl (pH 7.8), 10 mM EDTA, 50 mM NaCl, and centrifuged in a Beckman SW40 rotor at 35,000 rpm for 3.5 h. The DNA-containing fractions were pooled and digested for 1 h at 37ºC with DNase-free RNase A (100 µg/ml,Sigma). The solution was phenol-treated and the DNA ethanol-precipitated and dissolved in 10 mM Tris HCl, pH 8.0, 1 mM EDTA (TE).

Isolation and sequence determination of ASF virus DNA ends.

ASF virus DNA (8 µg) was treated with the restriction endonuclease BssHII (15 units, New England Biolabs) and alkaline phosphatase (5 units, Boehringer Mannheim). After 30 min at 37ºC the phosphatase was inactivated by treatment with 10 mM nitrilotriacetic acid and 0.5% sodium dodecylsulphate during 15 min at 68ºC. The sample was extracted with phenol-

chloroform, the aqueous phase passed through a Sephadex G-50 spun minicolumn and the DNA precipitated with ethanol. After labeling with [γ-^{32}P]-ATP (Amersham, 3000 Ci/mmol) and polynucleotide kinase (Boehringer Mannheim), the DNA was subjected to electrophoresis in a 12% acrylamide gel containing 50 mM Tris-borate buffer (pH 8.3) and 1 mM EDTA (15). The gel was wrapped in Saran-wrap, autoradiographed and portions of the gel containing the bands of interest were sliced and placed on a denaturing gel containing 7.6% acrylamide, 0.4% methylenebisacrylamide, 50% urea, 0.1 M Tris-borate (pH 8.3) and 2 mM EDTA and electrophoresed at 50ºC. The gel was autoradiographed, the bands were excised and the DNA was recovered by diffusion, filtered through a Sephadex G-50 spun minicolumn, precipitated with ethanol and sequenced by the Maxam and Gilbert technique (15).

Isolation of DNA from ASF virus-infected VERO cells

Cells were infected with ASF virus at a moi of 5 pfu per cell. At 48 hpi the cells were collected in Dulbecco's modified Eagle medium at 10^8 cells ml^{-1} and disrupted by sonication. The cell debris was removed by centrifugation. The supernatant (2 ml) was treated with 500 µg/ml proteinase K and 0.5% SDS for 30 min at 37ºC and extracted twice with phenol-Cl_3CH (10:1). The DNA was precipitated with ethanol, resuspended in TE and digested for 1 h at 37ºC with 100 µg/ml DNase-free RNase A. The DNA was extracted with phenol, ethanol precipitated and dissolved in TE.

DNA hybridization

The replicative form of a recombinant phage M13mp9 (16) containing the TIR sequence, was purified by the alkaline-SDS method (17). The DNA was labeled by nick-translations using [α-^{32}P]-dATP (Amersham, 400 Ci/mmol) and *E. coli* DNA polymerase (Boehringer Mannheim) (18). Hybridization was carried out in 5 x SSC at 65ºC. Filters were washed at the same stringency and autoradiographied.

RESULTS

Fast and slow components at the ends of ASF virus DNA.

An analysis of the nucleotide sequence at the left

terminal fragment Eco RI K' cloned in plasmid pBR325 (19), showed the existence of a BssHII site at about 100 bp from the end (A. González, V. Calvo and E. Viñuela, unpublished data). Presumably this site is also present at the other end, because

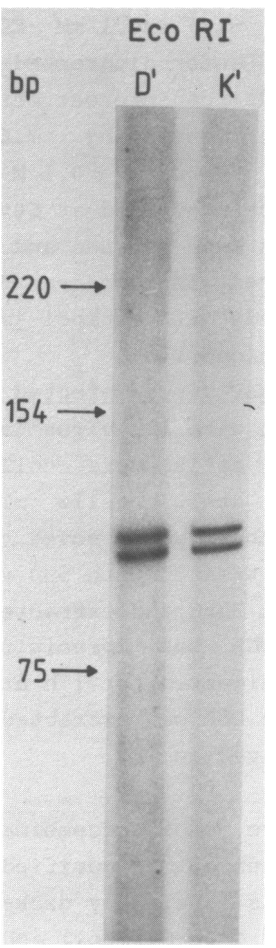

Figure 1. Fast and slow components in the terminal BssHII fragments of ASF virus DNA. ASF virus DNA was digested with EcoRI (New England Biolabs) and subjected to electrophoresis through a 0.8% low-melting temperature agarose gel. The terminal framents EcoRI K'(4.8 kb) and D' (9.8 kb) (4) were isolated from the gel by the method of Langridge et al (20), digested with BssHII, treated with alkaline phosphatase, labelled with [γ-^{32}P]-ATP and polynucleotide kinase and electrophoresed in a 12% acrylamide gel contaning 50 mM Tris-borate buffer, pH 8.3 and 1 mM EDTA (15).

of the terminal inverted repeat present in ASF virus genome. Figure 1 shows that a BssHII digestion of the terminal EcoRI fragments, K' and D', analyzed by non-denaturing polyacrylamide gel electrophoresis, gave place to two equimolar components of apparent size 90 and 100 bp (fast and slow components). When electrophoresed in denaturing gels, a single band in this size range was seen (data not shown). This behavior is similar to that found for vaccinia virus DNA ends (21).

Nucleotide sequence and structure of the fast and slow components of the terminal BssHII fragments of ASF virus DNA

Sequencing of the putative ASF virus DNA HPL was initiated from the BssHII site, after 5'-end labeling and neutral acrylamide electrophoresis. However, when the fragments were extracted from the gel and sequenced by the Maxam and Gilbert technique (15), the autoradiogram showed specific bands on a strong background that led to some ambiguities in the sequence. We assumed this was due to ^{32}P incorporated into nicks distributed among unpaired nucleotides in the HPL. To avoid this, slices containing the fast and slow components were rerun at denaturing conditions. The bands that had now the same electrophoretic mobility (data not shown) were extracted and sequenced. Figure 2 shows that, reading upward in the 5' to 3' direction, both components have the same sequence until the start of the underlined nucleotides, where the sequence diverged along a region of 37 nucleotides. Fig. 3A shows the sequence and the secondary structure with minimal energy (ΔG = -1.4 kcal mol^{-1}) (22) of the HPLs corresponding to the slow and fast components.

Fig. 3b shows that at 12 nucleotides from the ASF virus DNA HPL there is a 16 nucleotide-long sequence that had an homology greater than 80% with a sequence contiguous to the vaccinia virus HPL (21). Very close to the latter sequence there are in both viruses sequences of 11 nucleotides that, when compared with the same polarity, are complementary.

Structure of the ends of ASF virus DNA isolated from infected cells.

To study the nature of the ends of replicating ASF virus DNA molecules, DNA was isolated from either the virion or

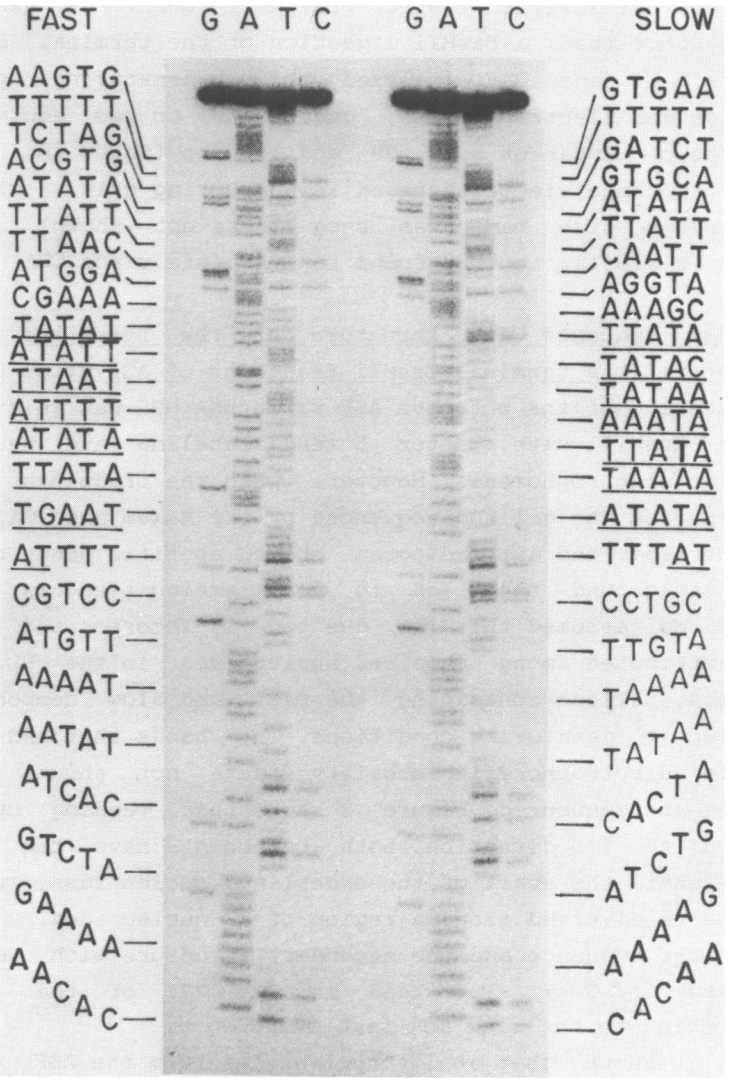

Figure 2. Nucleotide sequence of the fast and slow components of the terminal BssHII fragments of ASF virus DNA. Fast and slow components were labeled, isolated and sequenced as indicated in Materials and Methods.

virus-infected VERO cells and digested with the restriction endonuclease HindIII. After agarose electrophoresis the DNA fragments were transferred to a nitrocellulose membrane and hybridized to a ^{32}P-labeled terminal HindIII fragment that

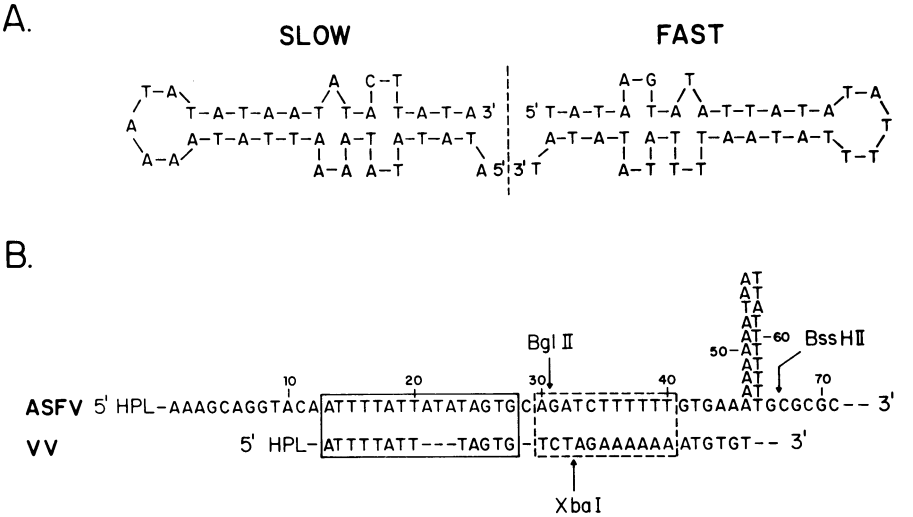

Figure 3. Structure of ASF virus DNA ends. A. Secondary structure of the fast and slow components in the HPLs. The HPLs start at the point of divergence in the sequence shown in Fig. 2. The structure of minimal energy (ΔG = -1.4 kcal/mol) was determined by Tinoco's rules (22). B. Nucleotide sequence of the regions adjacent ot the HPLs in ASF virus and VV DNAs. Residues boxed within continous lines indicate identities and those boxed with discontinuous lines indicate complementarity between ASF virus and VV sequences. The gap in the VV DNA sequence was introduced to achieve maximal alignment of the most conserved regions. The sequence from VV DNA is that published by Baroudy et al. (21).

included the 2.4 kb long TIR. Two bands of 2.7 and 8.8 kbp were detected when ASF virus DNA isolated from virions was examined in this manner and they corresponded to the terminal HindIII fragments (Fig. 4.1). The same two bands and two other bands twice the length of each terminal HindIII fragment were found using ASF virus DNA isolated from infected cells (Fig. 4.2). These results indicate the existence of head-to-head and tail-to-tail linked ASF virus DNA molecules in infected cells.

DISCUSSION

We have shown that both ASF virus DNA ends contain two components, present in about equal amounts, of apparent molecular length 90 (fast) and 100 (slow) base pairs, based in their mobility in neutral polyacrylamide gels. These

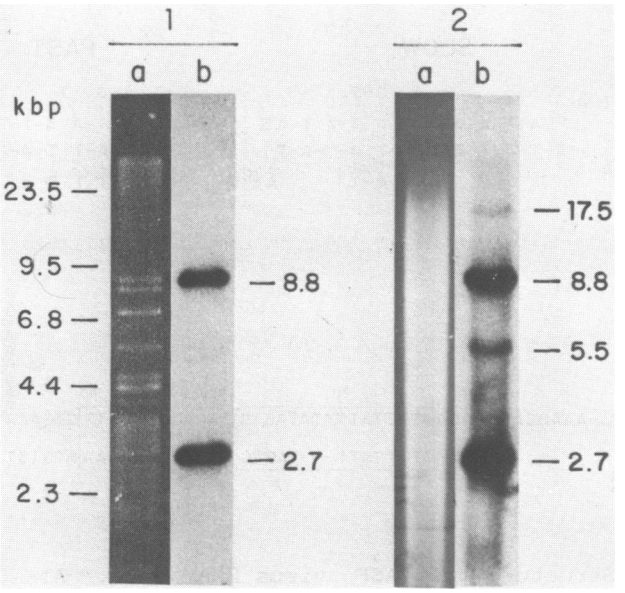

Figure 4. Dimeric terminal restriction fragments in DNA isolated from ASF virus-infected cells. 1. Viral DNA. a) DNA (0.8 µg) from purified virus particles was treated with HindIII (Boehringer Mannheim) and the digest was electrophoresed in 0.5% agarose with 0.5 µg/ml ethidium bromide; b) The HindIII fragments from a) were transferred to a nitrocellulose membrane and hybridized to a ^{32}P-labelled TIR sequence cloned in the phage M13mp9 (16). 2. DNA from infected cells . a) 5 µg of DNA were digested with HindIII and the digest was electrophoresed as indicated in 1. b) The HindIII fragments from a) were transferred to nitrocellulose and hybridized with the same probe as in 1.

components had the same electrophoretic mobility in an denaturing gel (not shown). These properties are similar to those of vaccinia virus DNA ends and suggested that the two strands in the fast and slow components were held together covalently and differed in sequence, but not in length, to give place to a HPL (21).

An analysis of the nucleotide sequence and the most stable secondary structure of the HPLs corresponding to the slow and fast components indicated that they were 37 nucleotide-long sequences composed, almost entirely, of incompletely paired A and T residues that, when examined in opposite polarities,

were inverted and complementary, as those found in vaccinia viruses (21).

At 12 nucleotides from the HPLs there is a 16 nucleotide-long sequence that is almost identical to a similar sequence contiguous to the HPL present in vaccinia virus DNA. This sequence could be a recognition signal for the nicking enzyme postulated in the Moss-Moyer model (21,23,24) to produce a 3'-hidroxyl end that could serve as a primer for DNA replication. One nucleotide from the last sequence there is, in both ASF and vaccinia virus DNA, an 11 nucleotide-long sequence that, when compared with the same polarity are complementary. The significance of this sequence is unclear.

An implication of this replication model for linear, double-stranded viral genomes is the existence of concatemeric DNA intermediates, formed by genomic units linked head-to-head and tail-to-tail. In agreement with this prediction, our results show the presence of double-size terminal restriction fragments (Fig. 4.2) in viral DNA isolated from infected cells.

Although the morphology of ASF virus is similar to that of vertebrate iridoviruses, ASF virus has more properties in common with poxviruses than with iridoviruses. These include the presence in the virion of enzymes for messenger RNA synthesis (7-10), the host RNA polymerase II-independent viral RNA synthesis in infected cells (J. Salas, M.L. Salas, and E. Viñuela, unpublished data), the presence of TIR in the genome (5) and, as reported here, the structure of DNA ends both in replicating and non-replicating molecules. Altogether, these properties suggest a phylogenetic relationship between ASF virus and the poxviruses, rather than convergent evolution of distinct functions.

ACKNOWLEDGEMENTS

We are grateful to P.M. Lizardi for his help in the computer analysis of the sequence. This work was supported by grants from Fondo de Investigaciones Sanitarias and Comisión Asesora para el Desarrollo de la Investigación Cientifica y Técnica to Inmunología y Genética Aplicada, S.A.

REFERENCES

1. Wardley, R.C., Hamilton, F., Wilkinson, P.J. (1979) Arch. Virol. 61, 217, 225.
2. Viñuela, E. (1985) Curr. Top. Microbiol. Immunol. 116, 151-170.
3. Enjuanes, L., Carrascosa, A.L. and Viñuela, E. (1976) J. Gen. Virol. 32, 479-492.
4. Almendral, J.M., Blasco, R., Ley, V., Beloso, A., Talavera, A. and Viñuela, E. (1984) Virology 133, 258-270.
5. Sogo, J.M., Almendral, J.M., Talavera, A. and Viñuela, E. (1984) Virology 133, 271-275.
6. Ortín, J., Enjuanes, L. and Viñuela, E. (1979) J. Virol. 31, 579-583.
7. Kuznar, J., Salas, M.L. and Viñuela, E. (1980) Virology 101, 169-175.
8. Salas, M.L., Kuznar, J. and Viñuela, E. (1981) Virology 113, 484-491.
9. Kuznar, J., Salas, M.L. and Viñuela, E. (1981) Arch. Virol. 96, 307-320.
10. Salas, M.L., Kuznar, J., and Viñuela, E. (1983) Arch. Virol. 77, 77-80.
11. Dales, A. and Pogo, B.G.T. (1981) Biology of poxvirus.In : Virology monographs (Kingsbury, D.W.and Zur Hausen, H. eds.), vol. 18, pp 1-19. Springer, Berlin, Heidelberg, New York.
12. Moss, B. (1985) Replication of Poxviruses. In : Virology (B.N. Fields et al. eds.), pp 685-703, Raven Press, New York.
13. Enjuanes, L., Carrascosa, A.L., Moreno, M.A. and Viñuela, E. (1976) J. Gen. Virol. 32, 471-477.
14. Black, D.N. and Brown, F. (1976) J. Gen. Virol. 32, 509-518.
15. Maxam, A.M. and Gilbert, W. (1980) Methods in Enzymol. (Grossman, L. and Moldave, K. eds.), vol. 65, pp 499-560, Academic Press, New York.
16. Messing, J. (1983) Methods in Enzymol. (Wu, R., Grossman, L. and Moldave, K. eds.), vol. 101, pp 20-78, Academic Press, New York.
17. Birnboin, H.C. and Doly, J. (1979) Nucl. Acids Res. 7, 1513-1523.
18. Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-256.
19. Ley, V., Almendral, J.M., Carbonero, P., Beloso, A., Viñuela, E. and Talavera, A. (1984) Virology 133, 249-257.
20. Langridge, J., Langridge, P. and Bergquist, P.L. (1980) Anal. Biochem. 103, 264-271.
21. Baroudy, B.M., Venkatesan, S. and Moss, B. (1983) Cell 28, 315-324.
22. Tinoco, I.,Jr., Borer, P.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C., Crothers, D.M. and Gralla, J. (1973) Nature New Biol. 246, 40-41.
23. Moyer, R.W. and Graves, R.L. (1981) Cell 27, 391-401.
24. Baroudy, B.M., Venkatesan, S. and Moss, B. (1983). Cold Spring Harbor Symp. Quant. Biol. 47, 723-729.