Molecular Cloning of African Swine Fever Virus DNA

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African swine fever virus DNA (about 170 kbp) was cleaved with the restriction endonuclease EcoRI and most of the resulting 31 fragments were cloned in either the phage vector λWES.MI or the plasmid pBR325. Three fragments were not cloned in those vectors, the largest fragment EcoRI-A (21.2 kbp) and the two crosslinked terminal fragments, EcoRI-K and D. Endonuclease SalI cut fragment EcoRI-A into three pieces which were cloned in plasmid pBR322. The two terminal EcoRI fragments were cloned after removal of the crosslinks with nuclease S1 and addition of EcoRI linkers to the fragment ends. The complete library of the cloned fragments accounted for about 98% of ASF virus genome, the missing sequences being those removed by the nuclease S1 in the process of cloning the terminal fragments.

INTRODUCTION

African swine fever (ASF) is an important disease of domestic pigs (Hess, 1971, 1981), produced by an icosahedral cytoplasmic deoxyvirus of about 200 nm (Breese and DeBoer, 1966). The genome of ASF virus is a linear duplex DNA with a molar mass of about $100 \times 10^{6}$ g mol$^{-1}$ (Enjuanes et al., 1976) and covalently closed ends (Ortín et al., 1979), similar to those present in poxvirus DNA (Geshelin and Berns, 1974; Baroudy et al., 1982).

The large size of ASF virus DNA and the low virus production in cell culture have made it difficult to study ASF virus at the molecular level. The availability of clones with defined DNA fragments would be useful for mapping viral transcripts and their translation products, sequencing regions of the viral genome, and for the study of gene expression.

This paper shows the molecular cloning of a collection of restriction EcoRI and SalI fragments of ASF virus DNA, which account for about 98% of the viral genome.

MATERIALS AND METHODS

Viruses and cells. ASF virus, adapted to grow in VERO cells, was described by Almendral et al. (1984).

DNAs. ASF virus DNA was isolated as described by Almendral et al. (1984).

Bacteriophage λWES.MI and phage recombinants were precipitated from Escherichia coli lysates as described (Yamamoto et al., 1970) and purified by either CsCl centrifugation or chromatography in Biogel A5M (Sain and Erdei, 1981).

Plasmids pBR322 and pBR325, with or without ASF virus DNA restriction fragments, were prepared by standard techniques (Maniatis et al., 1982). DNAs were stored in TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA).

DNA restriction, electrophoresis, and hybridization. Restriction enzyme digests, agarose electrophoresis, blotting, and hybridization were carried out as described by Maniatis et al. (1982).

Cloning of internal restriction fragments.
(1) Cloning in \( \lambda WES.\lambda B \). EcoRI restriction fragments were cloned in \( \lambda WES.\lambda B \) as described (Hohn and Murray, 1977; Hohn, 1979).

(2) Cloning in pBR325. Ligation of ASF virus DNA EcoRI fragments, recovered from agarose gels, to pBR325 cut with EcoRI was done by standard techniques (Maniatis et al., 1982). The ligation products were used to transform \( E.\) coli HB101 (Mandel and Higa, 1970) and the bacteria plated on L agar (L medium with 1.6% agar) containing ampicillin (50 \( \mu \)g/ml) and crystal violet (2 \( \mu \)g/ml). The dye accumulates in chloramphenicol-resistant bacteria, giving place to blue colonies, whereas bacteria carrying recombinant pBR325 produce white colonies (Proctor and Rownd, 1982). The latter were tested for chloramphenicol sensitivity and by hybridization to ASF virus [\( ^{32}\)P]DNA (Grünstein and Hogness, 1975). The positive colonies were recovered from the master plate and used to prepare recombinant plasmids.

(3) Cloning in pBR322. Sall and EcoRI- Sall restriction fragments were cloned in pBR322 treated with the corresponding enzymes. After transformation, the recombinants were tested for tetracycline resistance in the absence of Mgf" and by hybridization to ASF virus [\( ^{32}\)P]DNA (Maniatis et al., 1982).

Isolation and cloning of terminal EcoRI restriction fragments. Terminal EcoRI fragments were isolated, free of internal fragments, by using the snapback-S1 technique of Jaureguiberri (1977) and Wittek et al. (1977). In some experiments, the terminal fragments were separated from each other by centrifugation of the nuclease digestion mixture in a 5–20% NaCl gradient in a Beckman SW40 rotor at 30,000 rpm for 6 hr at 4° (Liu et al., 1980). The fractions containing each terminal fragment were pooled and the DNA was precipitated with ethanol. The precipitate was dissolved in S1 buffer and treated with S1 nuclease. The blunt-ended fragments, obtained by either method, were added to EcoRI linkers in the presence of 0.5 mM ATP (Ferretti and Sgaramella, 1981) and T4 DNA ligase. After EcoRI treatment, vector DNA (pBR325 or \( \lambda WES.\lambda B \) arms) was added as a carrier and the mixture was passed through a Ultrigel AcA 34 column (Anderson, 1981). The DNA in the void volume was treated with T4 DNA ligase in the presence of 1 mM ATP, and the recombinant molecules were packaged into lambda particles or used to transform \( E.\) coli HB101.

Recombinant nomenclature. Recombinants of pBR322 or pBR325 and a specific restriction fragment were named p2 (for pBR322) or p5 (for pBR325), followed by an abbreviation for the restriction enzyme (R, EcoRI; S, Sall), a letter designating the fragment and the isolation number of the recombinant. Recombinants of lambda DNA and a restriction fragment were named as above by substituting L for p2 or p5.

Recombinants with fragments produced by two nucleases were designated as above, by taking into account the orientation of the restriction fragment map (Almendral et al., 1984), indicating, first, the site cut to the left and then the one cut to the right, separated both by a tilted bar. Thus, for example, p2SD/RC7 (Fig. 7).

Materials. Restriction enzymes, \( E.\) coli DNA polymerase and T4 DNA ligase were purchased from New England Biolabs and nuclease S1 from Sigma Chemical Company. Spectinomycin was a gift from Upjohn Diagnostics and the other antibiotics were from Sigma Chemical Company. Agarose (type II) was from Sigma Chemical Company, nitrocellulose membranes (HAW 304FO) from Millipore, Ultrogel AcA34 from LKB, and Biogel A5m from BioRad. Putrescine and spermidine were purchased from Sigma Chemical Company and \([\gamma-^{32}\)P]ATP, \([\alpha-^{32}\)P]dATP, and \([\alpha-^{32}\)P]dTTP from the Radiochemical Center, Amersham.

RESULTS

Random Cloning of EcoRI Fragments

Cleavage of ASF virus DNA with EcoRI produces 31 fragments (Almendral et al., 1984). From 259 recombinants obtained, 254 contained one single fragment, the largest one being EcoRI-C (11.5 kbp) and the smallest EcoRI-K (4.8 kbp). Fragments EcoRI-L, N, P, R, and U, with sizes below
4.8 kbp, were obtained in recombinants carrying more than one viral fragment. Since fragments EcoRI-D' (10.7 kbp) and K' (4.8 kbp) are terminal (Almendral et al., 1984), recombinants with either one of those fragments were not obtained. The most frequent fragment cloned in λWES.AB was EcoRI-F (108 clones), which has a size of 8.4 kbp. Figure 1 shows the characterization of the recombinants carrying single EcoRI fragments.

To distinguish the recombinants with fragments that, being different, had the same electrophoretic mobility, [32P]DNA from one of these recombinants was hybridized to the DNA restriction fragments from each of the other recombinants. The clones giving a positive signal carried one of the two fragments and the negative ones the second fragment. This was confirmed by hybridization of [32P]DNA from one negative recombinant with the DNA restriction fragments from each of the other clones (data not shown).

Figure 1b shows the map position (Almendral et al., 1984) of the 16 EcoRI fragments cloned in λWES.AB in the experiment indicated above, and that of the terminal EcoRI-D', separately cloned in λWES.AB (see below).

Cloning of Isolated EcoRI Fragments

Internal EcoRI fragments not cloned as single inserts in phage λWES.AB were electroeluted from agarose gels, incubated with EcoRI linearized pBR325 and DNA ligase and the reaction products were used

![Figure 1](image)

**Fig. 1.** Characterization of EcoRI fragments cloned in λWES.AB. (a) Phage recombinants carrying single EcoRI fragments were purified and the DNA extracted and digested with EcoRI. The digests were subjected to electrophoresis in a 0.5% agarose gel. (b) The DNA was transferred from the gel shown in (a) to a nitrocellulose sheet and hybridized to ASF virus [32P]DNA. Lanes labeled ASFV were loaded with an EcoRI digest of ASF virus DNA. Other lanes are labeled with the letter designating the cloned EcoRI fragment. λA and λΩ indicate the two terminal EcoRI fragments of λWES.AB DNA.
to transform *E. coli* HB101. Figure 2 shows that all the expected fragments, except fragment *EcoRl*-A, were found in the recombinants.

Figure 7c shows the map position of the *EcoRl* fragments cloned in pBR325 and the terminal fragment *EcoRI*-K', separately cloned in pBR325 (see below).

**Cloning of Fragment *EcoRl*-A**

Two **SalI** sites divide fragment *EcoRl*-A in three segments, *EcoRl*-A/*SalI*-C (13.1 kbp), *SalI*-I′ (5.5 kbp), and *SalI*-B/*EcoRl*-A (3.0 kbp) (Almendral et al., 1984) (see Figs. 7a, d, e).

Fragment **SalI**-I′ was obtained after cloning a mixture of **SalI** fragments of ASF virus DNA into plasmid pBR322 (Fig. 3). In this experiment we also obtained, among others, fragment **SalI**-B (25 kbp), which contained the segment **SalI**-B/*EcoRl*-A (Figs. 3 and 7d, e).

To clone fragment *EcoRl*-A/*SalI*-C, fragment **SalI**-C, contaminated with fragments A to D was recovered from an agarose gel. The mixture was treated with *EcoRl* and the products ligated to a fragment of plasmid pBR322, flanked by the *EcoRl* and **SalI** cohesive sites. After transformation, we obtained, among others, the fragments *EcoRl*-A/*SalI*-C and **SalI**-B/
FlG. 3. Characterization of Snll fragments cloned in pBR322. (a) Recombinants carrying different Snll fragments were digested with nuclease Snll and the reaction products electrophoresed in parallel with a Snll digest of ASK virus DNA. (b) The DNA was transferred from the gel shown in (a) to a nitrocellulose sheet and hybridized to ASK virus [\textsuperscript{32}P]DNA. The lane labeled ASFV was loaded with a Snll digest of ASF virus DNA. Other lanes are labeled with the letter designating the cloned Snll fragments.

EcoRI-A (Figs. 4 and 7e) which, together with fragment Snll-I' (Fig. 3), covered the whole length of fragment EcoRI-A.

Cloning of the Terminal Fragments EcoRI-D' and K'

Fragments EcoRI D' (10.7 kbp) and K' (4.8 kbp), obtained by the snapback-S1 technique, were inserted into plasmid pBR325 after addition of EcoRI linkers and the products used to transform E. coli HB101. Figure 5a shows that all the recombinants obtained in this experiment contained viral inserts shorter than either terminal fragment. The recombinant with the largest insert (plasmid p5RD76) had a 4.4-kbp-long viral fragment (Fig. 5a) and hybridized to both terminal fragments EcoRI-D' and K' (Fig. 5a). This indicated that the recombinant contained, at least in part, the 2.1-kbp-long inverted terminal repetition present in ASK virus DNA (Sogo et al., 1984). The sensitivity of either p5RD76 plasmid or the other recombinants to Pvul endonuclease indicated that the inserts belonged to fragment EcoRI-D', since EcoRI-K' has no Pvul site (Almendral et al., 1984) (data not shown).

The terminal inverted repeat cloned in

FlG. 4. Characterization of recombinants containing Snll/EcoRI double restriction fragments. (a) pBR322 recombinants were digested with both Snll and EcoRI and the reaction products subjected to agarose electrophoresis, in parallel with HindIII fragments of lambda DNA as size markers; lane 1, recombinant p2RA/SC3; lane 2, p2RC'/SA5; lane 3, p2RD/SD4; lane 4, p2SD/RC7; lane 5, p2SB/RA1. (b) The DNA was transferred from the gel shown in (a) to a nitrocellulose sheet and hybridized to ASF virus [\textsuperscript{32}P]DNA; lanes 1-5 as in (a). (c) and (d) Nitrocellulose strips with immobilized EcoRI or Snll fragments, respectively, of ASF virus DNA were hybridized to [\textsuperscript{32}P]DNA from each recombinant (lanes 1-5) or from ASF virus (lane 0).
recombinant p5RD76 was used as a probe to select a new set of recombinants containing terminal fragments. One recombinant, p5RK1, contained a 3.8-kbp-long insert (Fig. 5b) that lacked PvuI sites indicating that it was a part of fragment EcoRI-K' which had lost about 1 kbp by the S1 treatment involved in the cloning process (data not shown).

To isolate an EcoRI-D' subfragment as complete as possible, the terminal cross-links of ASF virus DNA were removed without the snapback renaturation step. A mixture of EcoRI fragments (A to D'), isolated in a NaCl gradient, was treated with nuclease S1 and the blunt-ended products were cloned in λWES.XB after addition of EcoRI linkers. The largest recombinant (LRD'16) that hybridized with p5RD76 contained a 9.0-kbp-long insert, shorter than fragment EcoRI-D' (10.7 kbp). Figure 5c shows that LRD'16 hybridized with ASF virus [32P]DNA and the [32P]DNA from the recombinant hybridized with fragments EcoRI-D' and K'.

To determine the extent of the deletions at either end of the cloned EcoRI-D' fragment in recombinant LRD'16, we mapped the SmaI sites in LRD'16, knowing that SmaI cleaves EcoRI-D' (10.7 kbp) in two 7.0- and 3.7-kbp-long segments (Almendral et al., 1984), the left lambda arm in two 19.7- and 1.9-kbp-long segments and the right arm in two other segments with a length of 8.2 and 5.9 kbp (Daniels and Blattner, 1982). Figure 6 shows that digestion of LRD'16 DNA with SmaI produced four fragments. Two, 19.7 and 5.9 kbp long, derived from the ends of the molecule, respectively. A third fragment of 11.6 kbp contained the 8.2-kbp-long fragment from the right arm of λWES.XB DNA plus a 3.4-kbp-long piece from the insert. The fourth fragment of 7.5 kbp was the sum of the

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**Fig. 5.** Characterization of recombinants carrying terminal EcoRI fragments. (a) pBR325 recombinants with terminal EcoRI fragments were digested with EcoRI and the reaction products electrophoresed in parallel with HindIII fragments of lambda DNA. Lanes 1-4, recombinants p5RD76, p5RD61, p5RD48, and p5RD47, respectively; lane 5, immobilized EcoRI fragments of ASF virus DNA hybridized to ASF virus [32P]DNA; lane 6, immobilized EcoRI fragments of ASF virus DNA hybridized to p5RD76 [32P]DNA. (b) Lane 1, recombinant p5RK1 was digested with EcoRI and the reaction products were electrophoresed in parallel with HindIII fragments of lambda DNA; lane 2, DNA was transferred from the gel shown in lane 1 to a nitrocellulose sheet and hybridized to ASF virus [32P]DNA; lane 3, as lane a5; lane 4, immobilized EcoRI fragments of ASF virus DNA hybridized to p5RK1 [32P]DNA. (c) Lane 1, recombinant LRD'16 was digested with EcoRI and the reaction products were electrophoresed in parallel with HindIII fragments of lambda DNA; lane 2, DNA was transferred from the gel shown in lane 1 to a nitrocellulose sheet and hybridized to ASF virus [32P]DNA; lane 3, as lane a5; lane 4, immobilized EcoRI fragments of ASF virus DNA hybridized to LRD'16 [32P]DNA. p Indicates plasmid pBR325 and λA, λ9, and λ(A + Ω) the terminal EcoRI fragments, alone and linked through the cohesive ends of lambda DNA.
1.9-kbp-long fragment, derived from the left arm of λWES.AB DNA, and a 5.6-kbp-long piece from the insert. These results led to the conclusion that fragment EcoRI-D' in the recombinant LRD16 lacked 1.4 and 0.3 kbp in the left and right ends, respectively. A similar analysis done with nuclease PvuII and KpnI led to the same conclusion (data not shown).

**DISCUSSION**

A library of clones of all the EcoRI fragments of ASF virus DNA, except fragment A, has been obtained. Fragment EcoRI-A (21.2 kbp) was cloned in three pieces, obtained by digestion of EcoRI-A with nuclease SalI (Figs. 7d and e).

In total, about 98% of the ASF virus DNA...
DNA sequences (Almendral et al., 1984) have been inserted into either phage or plasmid vectors. The missing sequences were deletions produced by nuclease S1 in the process of cloning the terminal fragments; about 1 kbp in fragment EcoRI-K (4.8 kbp) and 1.7 kbp in EcoRI-D (10.7 kbp). The cloning of the terminal fragments of vaccinia virus DNA by similar methods, but using vaccinia endonuclease instead nuclease S1, produced deletions of about 50 kbp near the crosslinks (Wittek and Moss, 1980; Baroudy et al., 1982). The large difference of the deletions produced in each case was probably due to either the different nucleases used to remove the crosslinks or the presence of more nicks or gaps in ASF virus than in vaccinia virus DNA. However, Pickup et al. (1983) have cloned one vaccinia terminal fragment without any deletion.

The packaging limits of AWES.XB (Feiss et al., 1977; Williams and Blattner, 1981) indicate a cloning capacity of 2.1–15.1 kbp. Therefore, fragments EcoRI-B (14.5 kbp) to P (22 kbp) were expected to be cloned as single inserts in AWES.XB. However, neither fragment EcoRI-B nor single fragments shorter than EcoRI-K (4.8 kbp) were found among the recombinants. It is likely that the packaging system used selects against extreme-size inserts.

The clones described in this paper have been used for mapping restriction fragments (Almendral et al., 1984) and viral transcripts (M. L. Salas, J. Rey, J. M. Almendral, A. Talavera, and E. Vinuela, unpublished results) of ASF virus DNA.

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