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 λ WES. λ B 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 100×10^6 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 λ WES. λ B 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 λ WES. λ B. *EcoRI* restriction fragments were cloned in λ WES. λ 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 μ g/ml) and crystal violet (2 μ 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. *SalI* and *EcoRI*-*SalI* restriction fragments were cloned in pBR322 treated with the corresponding enzymes. After transformation, the recombinants were tested for tetracycline resistance in the absence of Mg^{2+} 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 Jaureguiberry (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 Sgarbetta, 1981) and T4 DNA ligase. After *EcoRI* treatment, vector DNA (pBR325 or λ WES. λ B arms) was added as a carrier

and the mixture was passed through a Ultrogel 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, *SalI*), 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 [γ - 32 P]ATP, [α - 32 P]dATP, and [α - 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 λ WES. λ B 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, [32 P]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 [32 P]DNA from one negative recombinant with the DNA restriction fragments from each of the other clones (data not shown).

Figure 7b shows the map position (Almendral *et al.*, 1984) of the 16 *EcoRI* fragments cloned in λ WES. λ B in the experiment indicated above, and that of the terminal *EcoRI*-D', separately cloned in λ WES. λ B (see below).

Cloning of Isolated *EcoRI* Fragments

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

FIG. 1. Characterization of *EcoRI* fragments cloned in λ WES. λ B. (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 [32 P]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. λ A and λ B indicate the two terminal *EcoRI* fragments of λ WES. λ B DNA.

to transform *E. coli* HB101. Figure 2 shows that all the expected fragments, except fragment *EcoRI*-A, were found in the recombinants.

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

Cloning of Fragment *EcoRI*-A

Two *SalI* sites divide fragment *EcoRI*-A in three segments, *EcoRI*-A/*SalI*-C (13.1 kbp), *SalI*-I' (5.5 kbp), and *SalI*-B/*EcoRI*-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/*EcoRI*-A (Figs. 3 and 7d, e).

To clone fragment *EcoRI*-A/*SalI*-C, fragment *SalI*-C, contaminated with fragments A to D was recovered from an agarose gel. The mixture was treated with *EcoRI* and the products ligated to a fragment of plasmid pBR322, flanked by the *EcoRI* and *SalI* cohesive sites. After transformation, we obtained, among others, the fragments *EcoRI*-A/*SalI*-C and *SalI*-B/

FIG. 2. Characterization of *EcoRI* fragments cloned in pBR325. (a) Plasmid recombinants carrying each of the cloned fragments were digested with *EcoRI* and subjected to electrophoresis. (b) The DNA was transferred from the gel shown in (a) to a nitrocellulose sheet and hybridized to ASF virus [32 P]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.

FIG. 3. Characterization of *SalI* fragments cloned in pBR322. (a) Recombinants carrying different *SalI* fragments were digested with nuclease *SalI* and the reaction products electrophoresed in parallel with a *SalI* digest of ASF virus DNA. (b) The DNA was transferred from the gel shown in (a) to a nitrocellulose sheet and hybridized to ASF virus [^{32}P]DNA. The lane labeled ASFV was loaded with a *SalI* digest of ASF virus DNA. Other lanes are labeled with the letter designating the cloned *SalI* fragments.

EcoRI-A (Figs. 4 and 7e) which, together with fragment *SalI*-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 p5RD'76) 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 ASF virus DNA (Sogo *et al.*, 1984). The sensitivity of either p5RD'76 plasmid or the other recombinants to *PvuI* endonuclease indicated that the inserts belonged to fragment *EcoRI*-D', since *EcoRI*-K' has no *PvuI* site (Almendral *et al.*, 1984) (data not shown).

The terminal inverted repeat cloned in

FIG. 4. Characterization of recombinants containing *SalI*/*EcoRI* double restriction fragments. (a) pBR322 recombinants were digested with both *SalI* 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 [^{32}P]DNA; lanes 1-5 as in (a). (c) and (d) Nitrocellulose strips with immobilized *EcoRI* or *SalI* fragments, respectively, of ASF virus DNA were hybridized to [^{32}P]DNA from each recombinant (lanes 1-5) or from ASF virus (lane 0).

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 p5RD'76, p5RD'61, p5RD'48, and p5RD'47, respectively; lane 5, immobilized *EcoRI* fragments of ASF virus DNA hybridized to ASF virus [32 P] DNA; lane 6, immobilized *EcoRI* fragments of ASF virus DNA hybridized to p5RD'76 [32 P] DNA. (b) Lane 1, recombinant p5RK'1 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 [32 P] DNA; lane 3, as lane a5; lane 4, immobilized *EcoRI* fragments of ASF virus DNA hybridized to p5RK'1 [32 P] 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 [32 P] DNA; lane 3, as lane a5; lane 4, immobilized *EcoRI* fragments of ASF virus DNA hybridized to LRD'16 [32 P] DNA. p Indicates plasmid pBR325 and λ A, $\lambda\Omega$, and $\lambda(A + \Omega)$ the terminal *EcoRI* fragments, alone and linked through the cohesive ends of lambda DNA.

recombinant p5RD'76 was used as a probe to select a new set of recombinants containing terminal fragments. One recombinant, p5RK'1, 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 λ WES. λ B after addition of *EcoRI* linkers. The largest recombinant (LRD'16) that hybridized with p5RD'76 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 [32 P]DNA and the [32 P]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 λ WES. λ B DNA plus a 3.4-kbp-long piece from the insert. The fourth fragment of 7.5 kbp was the sum of the

FIG. 6. Determination of the deletion extents of recombinant LRD'16 carrying the terminal fragment *EcoRI*-D'. (a) Recombinant LRD'16 DNA was digested with *SmaI* and electrophoresed in parallel with *HindIII* fragments of lambda DNA. (b) DNA map of recombinant LRD'16, showing the *EcoRI* (R) and *SmaI* (M) sites (1), compared with the map of the terminal fragment *EcoRI*-D' (2) as determined by Almendral *et al.* (1984). TIR indicates the 2.1-kbp-long terminal inverted repetition of ASF virus DNA (Sogo *et al.*, 1984).

1.9-kbp-long fragment, derived from the left arm of λ WES. λ B DNA, and a 5.6-kbp-long piece from the insert. These results led to the conclusion that fragment *EcoRI*-D' in the recombinant LRD'16 lacked 1.4 and 0.3 kbp in the left and right ends, respectively. A similar analysis done with nuclease *PvuI* 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

FIG. 7. Map location of cloned fragments of ASF virus DNA. (a) Restriction site map of ASF virus DNA showing the *EcoRI* (I) and *SalI* (I) restriction sites (Almendral *et al.*, 1983); (b) location of the *EcoRI* fragments cloned in λ WES. λ B; (c) location of the *EcoRI* fragments cloned in pBR325; (d) location of the *SalI* fragments cloned in pBR322; (e) location of the *SalI*/*EcoRI* fragments cloned in pBR322.

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 of nuclease S1, produced deletions of about 50 bp 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 λ WES.8B (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 (2.2 kbp) were expected to be cloned as single inserts in λ WES.8B. 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. Viñuela, unpublished results) of ASF virus DNA.

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