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Cross-Links in African Swine Fever Virus DNA

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African swine fever virus DNA sediments in neutral sucrose density gradients as a single component with a sedimentation coefficient of 60S. In alkaline sucrose density gradients, this material shows two components with sedimentation coefficients of 85S and 95S, respectively. The sedimentation rate value of alkali-denatured virus DNA in neutral sucrose density gradients and the renaturation velocity of denatured DNA show that it reassociates much faster than expected from its genetic complexity. This behavior is compatible with the existence of interstrand cross-links in the molecule. We also present results which suggest that there are only a few such cross-links per molecule, that they are sensitive to S1 nuclease digestion, and that they are probably located next to the ends of the DNA.

African swine fever (ASF) virus is the causative agent of a highly contagious and frequently fatal disease of domestic swine (11), although chronic infections are becoming increasingly important in nature (4). The virus is icosahedral in shape, multiplies in the cytoplasm of the infected cell (2), and matures by budding through the plasma membrane (3, 14).

The genome of ASF virus consists of a double-stranded DNA molecule with a molar mass of $\sim 100 \times 10^6$ g/mol (7), whose replication in the infected cell requires the presence of the cell nucleus (15). Several properties of ASF virus DNA, such as sedimentation coefficient, molecular length, and $C_0t_{1/2}$ value, have already been reported (7). Virus DNA replication takes place after the induction in the infected cell of a DNA polymerase activity (20) susceptible to inhibition by phosphonoacetic acid (13).

We show in this paper that ASF virus DNA can be isolated from virions as a rapidly renaturing molecule, probably due to the existence of cross-links at or near the ends of the molecule. Several properties of ASF virus DNA are shown to be similar to those reported for vaccinia virus DNA (1, 10, 12, 16) and for the *Molluscum contagiosum* virus genome (17).

MATERIALS AND METHODS

Cells and virus. Vero cells (CCL 81) were obtained from the American Type Culture Collection and grown in the Dulbecco modification of Eagle minimal essential medium (5), containing 10% calf serum. The source and plaque assay of the ASF virus were described by Enjuanes et al. (6). Bacteriophage T4 was grown, labeled, and purified as described previously (7).

Reagents and enzymes. Media and sera were obtained from GIBCO Laboratories, Grand Island, N.Y. [*methyl*-^{3}H]thymidine (42 to 48 Ci/mmol) and [1-^{14}C]thymidine (50 to 56 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, England. Hydroxylapatite was Hypatite C from Clarkson Chemical Co.; Sarkosyl and *Aspergillus* S1 nuclease were obtained from Sigma Chemical Co., St. Louis, Mo.; and proteinase K, polyethylene glycol 6000, and sucrose were from E. Merck AG, Darmstadt, Germany. All other chemicals were analytical grade.

Labeling and purification of the virus. Subconfluent Vero cell cultures were infected with ASF virus at a multiplicity of infection of about 0.2 PFU/cell. After an adsorption period of 2 h at 37°C, the cells were washed free of inoculum virus and incubated in the Dulbecco modification of Eagle minimal essential medium supplemented with 0.5% calf serum and 4 μCi of [*methyl*-^{3}H]thymidine per ml. After 44 to 48 h, when approximately 50% of the cells had detached from the plates, the culture media were collected and the cells and cell debris were pelleted for 10 min at 2,500 rpm and 4°C in a Sorvall GS3 rotor. The extracellular virus was concentrated by precipitation with 6% (wt/vol) polyethylene glycol as described previously (7). The pellet was suspended in 0.01 M NaCl-0.001 M EDTA-0.01 M Tris-hydrochloride (pH 7.5), and a hydroxylapatite slurry in 1.0 M phosphate buffer (pH 6.8) was added to the suspension to give a phosphate concentration of 0.2 M. After 10 min at 0°C, the suspension was centrifuged for 5 min at 2,500 rpm and 4°C in a Sorvall SS-34 rotor. The supernatant was layered onto a discontinuous gradient of 50, 35, and 25% (wt/vol) sucrose layers in 0.01 M NaCl-0.001 M EDTA-0.01 M Tris-hydrochloride (pH 7.5) and centrifuged for 1.5 h at 25,000 rpm and 4°C in a Beckman SW40 rotor. The virus band, located in the interphase of the 50 and 35% sucrose layers, was collected, and the virus was pel-

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leted by high-speed centrifugation as described above.

DNA extraction. The DNA from either purified ASF virus or mixtures of purified T4 phage and ASF virus was extracted according to one of the following procedures. (i) DNA extraction at neutral pH was accomplished by resuspension of virus pellets in 0.01 M NaCl-0.01 M EDTA-0.01 M Tris-hydrochloride (pH 8.0)-0.5% Sarkosyl-500 μg of proteinase K per ml at 0°C and incubation overnight at 4°C. Virus DNA was then isolated by preparative neutral sucrose density gradient centrifugation as described below. (ii) Alkaline extraction was performed by resuspension of virus pellets at 0°C in 0.2 M NaOH-2% Sarkosyl-0.03 M 2-mercaptoethanol and overnight incubation at 4°C.

Sedimentation conditions. DNA was sedimented in 5 to 20% (wt/vol) linear sucrose density gradients in either 0.05 M NaCl-0.01 M EDTA-0.01 M Tris-hydrochloride (pH 7.5) or 0.7 M NaCl-0.3 M NaOH-0.01 M EDTA. Centrifugation was at 30,000 rpm and 20°C in a Beckman SW40 rotor for the times indicated in the corresponding figure legends.

S1 nuclease treatment. Treatment with S1 nuclease was performed in a buffer containing 0.3 M NaCl, 5×10^{-4} M $ZnSO_4$, 0.03 M sodium acetate, and 0.5 μg of calf-thymus DNA per ml (pH 4.6) under the conditions described below for each experiment.

Determination of radioactivity. The radioactivity of individual samples was determined in an Intertechnique liquid scintillation spectrometer after precipitation with 10% trichloroacetic acid in the presence of 50 μg of bovine serum albumin per ml as carrier. The counter data were processed for background and spillover in a PDP-11 computer.

RESULTS

Sedimentation rate of ASF virus DNA through neutral and alkaline sucrose density gradients. As previously shown (7), sedimentation of ASF virus DNA through neutral sucrose density gradients revealed a single peak with a sedimentation coefficient slightly smaller than that of T4 phage DNA (Fig. 1A). However, the results obtained after sedimentation through alkaline sucrose density gradients were highly dependent upon the experimental conditions used for virus growth and purification and for DNA extraction. When ASF virus was labeled after a high-multiplicity infection or when virus preparations were incubated at 37°C or stored at 4°C, the virus DNA obtained was nicked. However, the procedure described above for virus purification and virus DNA extraction largely circumvented this problem (Fig. 1B). A large proportion of virus DNA sedimented as two main peaks moving at approximately 85S and 95S when compared with the 73S T4 phage DNA marker (21, 22). This result was obtained both when purified virions were lysed under neutral or alkaline conditions and sedimented through alkaline sucrose density gradients. When virus DNA was extracted under neutral conditions and sedimented in alkali as described above, similar results were obtained.

The sedimentation coefficient of ASF virus

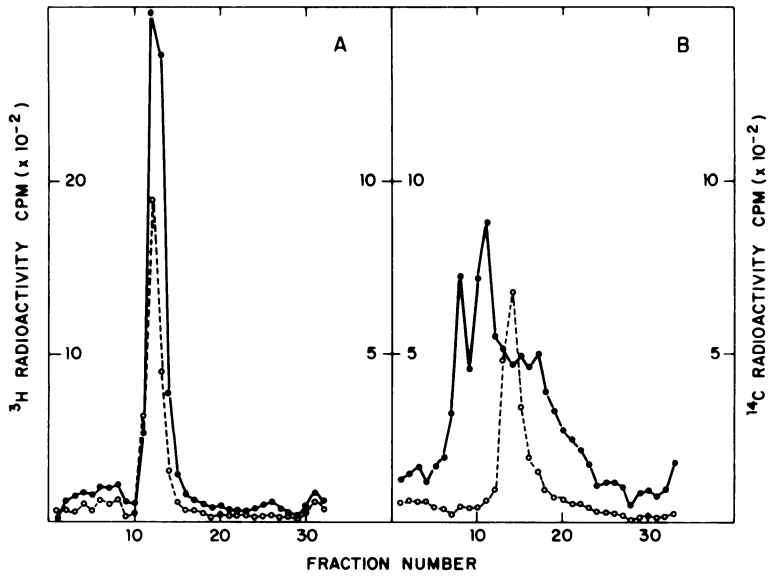

FIG. 1. *Sedimentation rate of ASF virus DNA through neutral and alkaline sucrose density gradients. The DNA of mixtures of ^{14}C-labeled T4 phage and ^{3}H-labeled ASF virus was extracted under neutral or alkaline conditions and sedimented for 3.3 h through neutral (A) or alkaline (B) sucrose density gradients, respectively, as described in the text. Symbols: ●, ASF virus [^{3}H]DNA; ○, T4 phage [^{14}C]DNA. In this and subsequent gradients, the direction of sedimentation is to the left.*

DNA in alkali (Fig. 1B) was consistent with a single-stranded DNA with a molecular length twice that expected for virus DNA. The 85S peak could be a linear form and the 95S peak could be a circular one, derived from a DNA molecule with one or two terminal interstrand cross-links, respectively.

Reassociation of intact ASF virus DNA. If the structure suggested above is correct, intact denatured ASF virus DNA should reassociate very rapidly, since nucleation would not be a limiting step in the renaturation process (23). To test this prediction, a mixture of T4 phage [^{14}C]DNA and ASF virus [^{3}H]DNA was denatured in alkali and sedimented through a neutral sucrose density gradient. Since neutral sucrose gradients contained only 0.05 M NaCl, T4 phage DNA sedimented about 40% faster than in alkaline conditions (21). However, ASF virus DNA did not sediment as a double peak, but instead sedimented as a single, although somewhat broader, band (Fig. 2). The rate at which ASF virus DNA sedimented under these conditions was similar to that of double-stranded DNA, as tested by sedimenting native ASF virus DNA in a parallel gradient, suggesting that renaturation took place during the sedimentation process.

Since the above experiment did not show the double-stranded nature of the alkali-denatured virus DNA after neutralization, the effect of S1 nuclease on such DNA was tested. A mixture of T4 phage [^{14}C]DNA and ASF virus [^{3}H]DNA was denatured in 0.2 M NaOH and then either neutralized with HCl or boiled in alkali and neutralized. Both samples were treated with increasing concentrations of S1 nuclease, and the trichloroacetic acid-insoluble radioactivity was determined. Figure 3 shows that, as expected, T4 phage DNA was fully degraded, irrespective of whether it was intact or fragmented. On the contrary, denatured and neutralized intact ASF virus DNA was 60 to 70% resistant to S1 nuclease digestion, and this resistance was completely lost when virus DNA was fragmented before neutralization. These results are consistent with the structure proposed above and further indicate that the number of cross-links that held together both viral DNA strands must be fairly small (up to 10 to 20 cross-links per molecule), since fragmented virus DNA (fragment size, ~500 base pairs) is almost as sensitive to S1 nuclease digestion as is control T4 phage DNA.

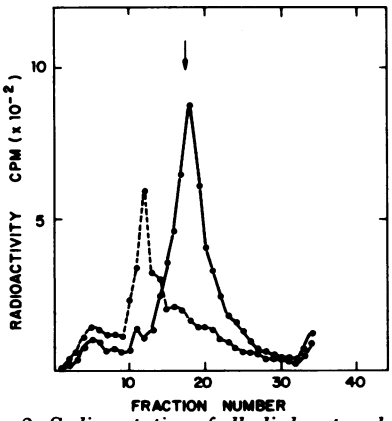

FIG. 2. *Sedimentation of alkali-denatured ASF virus DNA through neutral sucrose density gradient. A mixture of alkali-denatured T4 phage [^{14}C]DNA and ASF virus [^{3}H]DNA was sedimented for 2.5 h through a neutral sucrose density gradient as described in the text. Symbols: ●, ASF virus [^{3}H]DNA; ○, T4 phage [^{14}C]DNA. The arrow shows the position of native ASF virus DNA.*

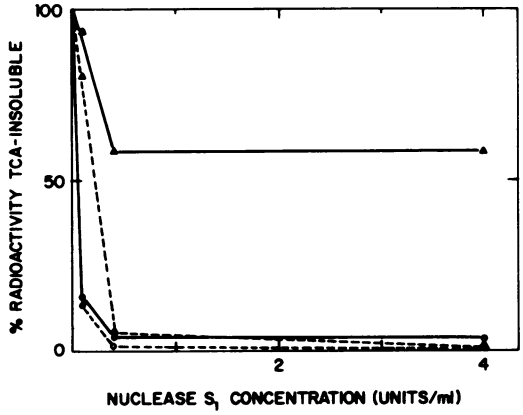

FIG. 3. *Treatment of alkali-denatured ASF virus DNA with S1 nuclease. Mixtures of T4 phage [^{14}C]-DNA and ASF virus [^{3}H]DNA were denatured in 0.2 M NaOH and then either neutralized with HCl or fragmented by boiling for 15 min in 0.2 M NaOH and then neutralized as described above. The samples were incubated for 2 h at 45°C with the concentrations of S1 nuclease indicated in the figure. Symbols: △, intact T4 phage [^{14}C]DNA; ○, fragmented T4 phage [^{14}C]DNA; ▲, intact ASF virus [^{3}H]DNA; ●, fragmented ASF virus [^{3}H]DNA.*

Sensitivity of the cross-links of ASF virus DNA to S1 nuclease. As will be discussed below, one of the structures that would be compatible with the results presented so far would be a double-stranded DNA whose ends are connected by terminal single-strand loops. In that case, the loops should be sensitive to digestion with S1 nuclease. To test this prediction, native ASF virus DNA was treated with S1 nuclease under conditions which would not induce single-strand breaks in native T4 phage DNA. Figure 4 shows the results obtained by sedimentation of ASF virus DNA in alkaline sucrose density gradients after digestion with S1 nuclease. Increasing concentrations of the enzyme converted

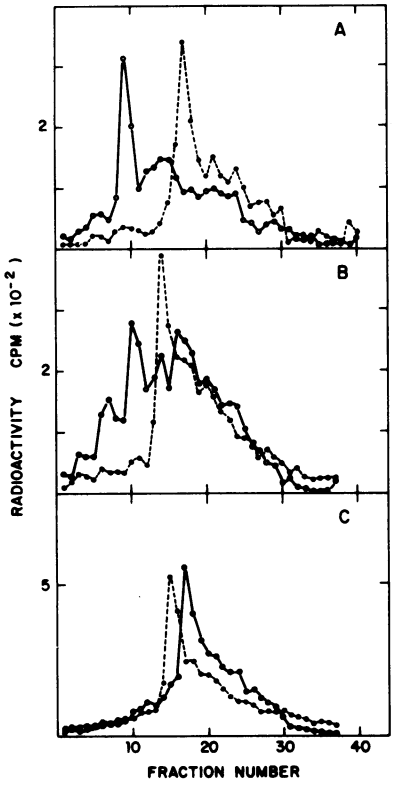

FIG. 4. *Sensitivity of the cross-links of ASF virus DNA to digestion with S1 nuclease. Native ASF virus [^{3}H]DNA was treated for 5 min at 37°C with the following amounts of S1 nuclease: (A) control without added enzyme; (B) 0.026 U; (C) 2.6 U. After incubation, EDTA was added to give a final concentration of 0.03 M, and the samples were denatured in 0.2 M NaOH and centrifuged for 3.3 h in alkaline sucrose density gradients as described in the text. Symbols: ○, ASF virus [^{3}H]DNA; ●, T4 phage [^{14}C]DNA.*

the fast-sedimenting forms of virus DNA (Fig. 4A) to a form with a sedimentation coefficient slightly smaller than that of T4 phage DNA, consistent with the length of ASF virus DNA (Fig. 4B and C). These results indicate that the cross-links in ASF virus DNA are sensitive to digestion with single-strand-specific nuclease.

DISCUSSION

ASF virus DNA, despite sedimenting as a single 60S component in neutral sucrose density gradients (7), contains two fast-sedimenting forms, 85S and 95S, when analyzed under alkaline conditions (Fig. 1). The sedimentation rate of the 85S form corresponds to a single-stranded DNA of ~120 μm in length, twice the value described for native ASF virus DNA (7). The 95S form could be a circular conformation of the 85S form. This suggestion, similar to that proposed for vaccinia virus DNA (1, 10, 12, 16), implies the existence of one or several interstrand cross-links in ASF virus DNA. A possible role of RNA or protein in the maintenance of such a link(s) has been ruled out, since incubation in alkali or with proteinase K does not affect the proportion of fast-sedimenting forms in the virus DNA population (Fig. 1B).

The structure proposed above is further supported by the evidence shown in Fig. 2 and 3. A fast reassociation rate would be a specific feature of a DNA having such a structure. In fact, Fig. 2 shows that neutralization of alkali-denatured ASF virus DNA leads to a form sedimenting as native virus DNA, whereas neutralization of alkali-denatured T4 phage DNA does not. Furthermore, intact denatured ASF virus DNA becomes resistant to S1 nuclease upon neutralization, whereas T4 phage DNA remains fully sensitive (Fig. 3). In addition, fragmentation of ASF virus completely abolishes this resistance (Fig. 3), suggesting that the number of cross-links per molecule is small.

Finally, the results shown in Fig. 4 indicate that the interstrand cross-links in ASF virus DNA are sensitive to single-strand-specific nuclease and suggest that they are located near the ends of the DNA molecule, since otherwise the product of S1 nuclease digestion would not give rise, upon denaturation, to unit-length virus DNA.

Therefore, we propose that the ASF virus genome consists of a continuous polynucleotide chain which is base paired to form a linear, duplex DNA molecule with single-strand loops at its ends.

The structure suggested for the termini of ASF virus DNA could have an important role in virus DNA replication or packaging or both. If the cross-links were not removed upon infection, DNA replication could not be semiconservative. Vaccinia virus DNA cross-links are removed shortly after infection (18), and their appearance in progeny virus DNA is one of the latest events in DNA maturation, long after virus DNA is fully replicated (9, 19). Vaccinia virus DNA termini have been proposed to be origins of virus DNA replication (8), but the possible role of the terminal cross-links in the process is not yet clear.

Information on the ASF virus DNA replication process or on the fate of virus DNA upon infection is scanty; therefore, more work is needed before the function(s) of the virus DNA terminal cross-links is understood.

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