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Replication of African Swine Fever Virus DNA in Infected Cells

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We have examined the ultrastructural localization of African swine fever virus DNA in thin-sections of infected cells by *in situ* hybridization and autoradiography. Virus-specific DNA sequences were found in the nucleus of infected Vero cells at early times in the synthesis of the viral DNA, forming dense foci localized in proximity to the nuclear membrane. At later times, the viral DNA was found exclusively in the cytoplasm. Electron microscopic autoradiography of African swine fever virus-infected macrophages showed that the nucleus is also a site of viral DNA replication at early times. These results provide further evidence of the existence of nuclear and cytoplasmic stages in the synthesis of African swine fever virus DNA. On the other hand, alkaline sucrose sedimentation analysis of the replicative intermediates synthesized in the nucleus and cytoplasm of infected macrophages showed that small DNA fragments (~6–12S) were synthesized in the nucleus at an early time, whereas at later times, larger fragments of ~37–49S were labeled in the cytoplasm. Pulse–chase experiments demonstrated that these fragments are precursors of the mature cross-linked viral DNA. The formation of dimeric concatemers, which are predominantly head-to-head linked, was observed by pulsed-field electrophoresis and restriction enzyme analysis at intermediate and late times in the replication of African swine fever virus DNA. Our findings suggest that the replication of African swine fever virus DNA proceeds by a *de novo* start mechanism with the synthesis of small DNA fragments, which are then converted into larger size molecules. Ligation or further elongation of these molecules would originate a two-unit concatemer with dimeric ends that could be resolved to generate the genomic DNA by site-specific nicking, rearrangement, and ligation as has been proposed in the *de novo* start model of Baroudy *et al.* (B. M. Baroudy, S. Venkatesam, and B. Moss, 1982, *Cold Spring Harbor Symp. Quant. Biol.* 47, 723–729) for the replication of vaccinia virus DNA.

INTRODUCTION

African swine fever virus (ASFV) is a large enveloped deoxyvirus with an icosahedral morphology that causes a severe disease in domestic pigs (Viñuela, 1985). Its genome is a double-stranded DNA molecule of ~170 kb that contains, like the DNA of poxviruses (Baroudy *et al.,* 1982a; Wittek and Moss, 1980), hairpin loops and terminal inverted repetitions (Sogo *et al.,* 1984; González *et al.,* 1986). Also like poxviruses, ASFV particles contain the enzymes required for early RNA synthesis and processing (Kuznar *et al.,* 1980; Salas *et al.,* 1981, 1986). Morphologically, however, ASFV is very similar to the iridoviruses that infect vertebrates (Carrascosa *et al.,* 1984). ASFV is one of the most complex animal viruses, and the analysis of the genome sequence has revealed the existence of 151 potential genes (Yáñez *et al.,* 1995).

Although ASFV has been considered for many years as a virus that multiplies exclusively in the cytoplasm of infected cells, previous results from our laboratory, obtained by autoradiography and *in situ* hybridization with light microscopy of infected cells, suggested that the replication of the viral DNA is initiated in the nucleus. This nuclear stage is followed by a phase of replication in the cytoplasm (García-Beato *et al.,* 1992b). However, the events in the replication of the viral DNA that occur in the nucleus are unknown at present. Also unknown is the mechanism by which the ASFV DNA is replicated. Head-to-head and tail-to-tail linked DNA molecules, which may correspond to concatemeric viral intermediates, have been detected in ASFV-infected Vero cells at late postinfection times (González *et al.,* 1986; Caeiro *et al.,* 1990), but no other data concerning the mode of ASFV DNA synthesis *in vivo* are available.

In the present work, we provide further evidence of the existence of a nuclear stage in the replication of ASFV DNA by determining the ultrastructural localization of the viral DNA in thin sections of infected cells by *in situ* hybridization and autoradiography. On the other hand, we have analyzed the replicative intermediates that are synthesized in the nucleus and cytoplasm of ASFV-infected cells. Sedimentation analysis of replicating viral DNA in alkaline sucrose gradients has shown that small DNA fragments are pulse-labeled in the nucleus at early times in the replication of the viral DNA, whereas larger molecules are synthesized in the cytoplasm at later

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times. Pulse–chase experiments showed that these cytoplasmic intermediates are precursors of the mature cross-linked viral DNA. In addition, the formation of dimeric concatemers, which are predominantly head-to-head linked, has been detected by pulsed-field electrophoresis and restriction enzyme analysis. In the light of these findings, we propose a model for the replication of ASFV DNA based in the *de novo* start model of Baroudy *et al.* (1982b) for the replication of vaccinia virus DNA.

RESULTS

Ultrastructural localization of ASFV DNA in infected Vero cells determined by *in situ* hybridization

The electron microscopic study on the intracellular localization of ASFV DNA was initiated by detecting virus-specific DNA sequences in infected cells by *in situ* hybridization. For this, we used Vero cells instead of porcine macrophages, which are the target cells in natural infections by the virus, because, as suggested by previous data (García-Beato *et al.,* 1992b), the infection appears to be better synchronized in Vero cells than in macrophage preparations. Preconfluent cultures of Vero cells were mock-infected or infected with ASFV and, at different times postinfection, they were fixed and processed for electron microscopy as described under Materials and Methods. Thin sections of these samples were hybridized with virus DNA labeled with digoxigenin, which was detected with an antidigoxigenin antibody, and then visualized through secondary labeling with an antibody coupled to 10-nm gold particles. As can be seen in Fig. 1A, mock-infected cells showed, after silver enhancing of the gold grains for an easier examination of the samples, a low background. In infected cells at early times in the replication of the viral DNA (6–8 h), the hybridization signal was predominantly localized in the nucleus (Fig. 1B). The signal is detected as a dense focus that occupies a relatively small area localized in proximity to the nuclear membrane as was also seen the nuclear signal in Vero cells by light microscopy (García-Beato *et al.,* 1992b). At later times, the grains were mainly found in the cytoplasm (Figs. 1C–1E). The cytoplasmic foci are constituted by more disperse grains but occupy a larger area than the nuclear signal. When virus morphogenesis is under way, the assembling virus particles are localized in these cytoplasmic areas (Fig. 1E). Figure 1D also shows the large clusters of mitochondria that surround the viral factories that contain assembling particles as has been previously described (Rojo *et al.,* 1998).

A statistical analysis of the distribution of the hybridization signal in the nucleus and cytoplasm of infected cells, based in the observation of a large number of cell sections, showed that at 6 h p.i., the signal was localized in the nucleus in ~90% of the cells (Fig. 2). At 8 h, this percentage fell to ~70% and then continued to decrease. At ≥12 h p.i., essentially all the cells showed the signal in the cytoplasm. These results are in agreement with previous data on the distribution of the hybridization signal in Vero cells obtained by light microscopy (García-Beato *et al.,* 1992b).

Localization of newly synthesized ASFV DNA in infected macrophages by electron microscopic autoradiography

The results described so far indicated that virus-specific DNA sequences are present in the nucleus of infected cells at early postinfection times. To confirm that replication of ASFV DNA also occurs in the nucleus, we used porcine alveolar macrophages because Vero cells can not be employed for this purpose because of the extensive cellular DNA synthesis that occurs in these cells. By contrast, alveolar macrophages synthesize DNA only to a small extent, and in addition, this low synthesis can be strongly inhibited, by 97%, in the presence of 2 mM sodium butyrate (García-Beato *et al.,* 1992b). Mock- and ASFV-infected macrophages cultured in the presence of 2 mM sodium butyrate were pulse-labeled with [^{3}H]thymidine at different times postinfection and then processed for electron microscopic autoradiography as described under Materials and Methods. The results are illustrated in Fig. 3. Mock-infected cells showed essentially no background grains (Fig. 3A), whereas infected macrophages presented, at early times in the replication of the virus DNA, significant label in the nucleus (Figs. 3B and 3C). This nuclear signal was usually localized near the nuclear membrane as was also detected the hybridization signal in the nucleus of infected Vero cells (see Fig. 1B). Infected macrophages with grains both in the nucleus and cytoplasm could also be observed at these times (Fig. 3D). In these cases, the nuclear focus was smaller than the cytoplasmic signal. Some grains were frequently seen across the nuclear membrane in these cells following a tract of electron dense material that joins the nuclear and cytoplasmic foci. These images might represent the replicating viral DNA being released from the nucleus and the ensuing cytoplasmic replication. At later postinfection times, the label was predominantly found in the cytoplasm occupying a large area (Fig. 3E). These findings at the electron microscopy level confirm that the nucleus is a site of viral DNA synthesis.

Sedimentation analysis of replicating ASFV DNA in alkaline sucrose gradients

To examine the events in the replication of ASFV DNA that occur in the nucleus and cytoplasm of infected cells as well as to gain information on the mechanism of synthesis of the viral DNA, we analyzed, as a first approach, the replicative intermediates that are synthesized in the nucleus and cytoplasm by alkaline sucrose sedimentation. In this study, we used again the macro-

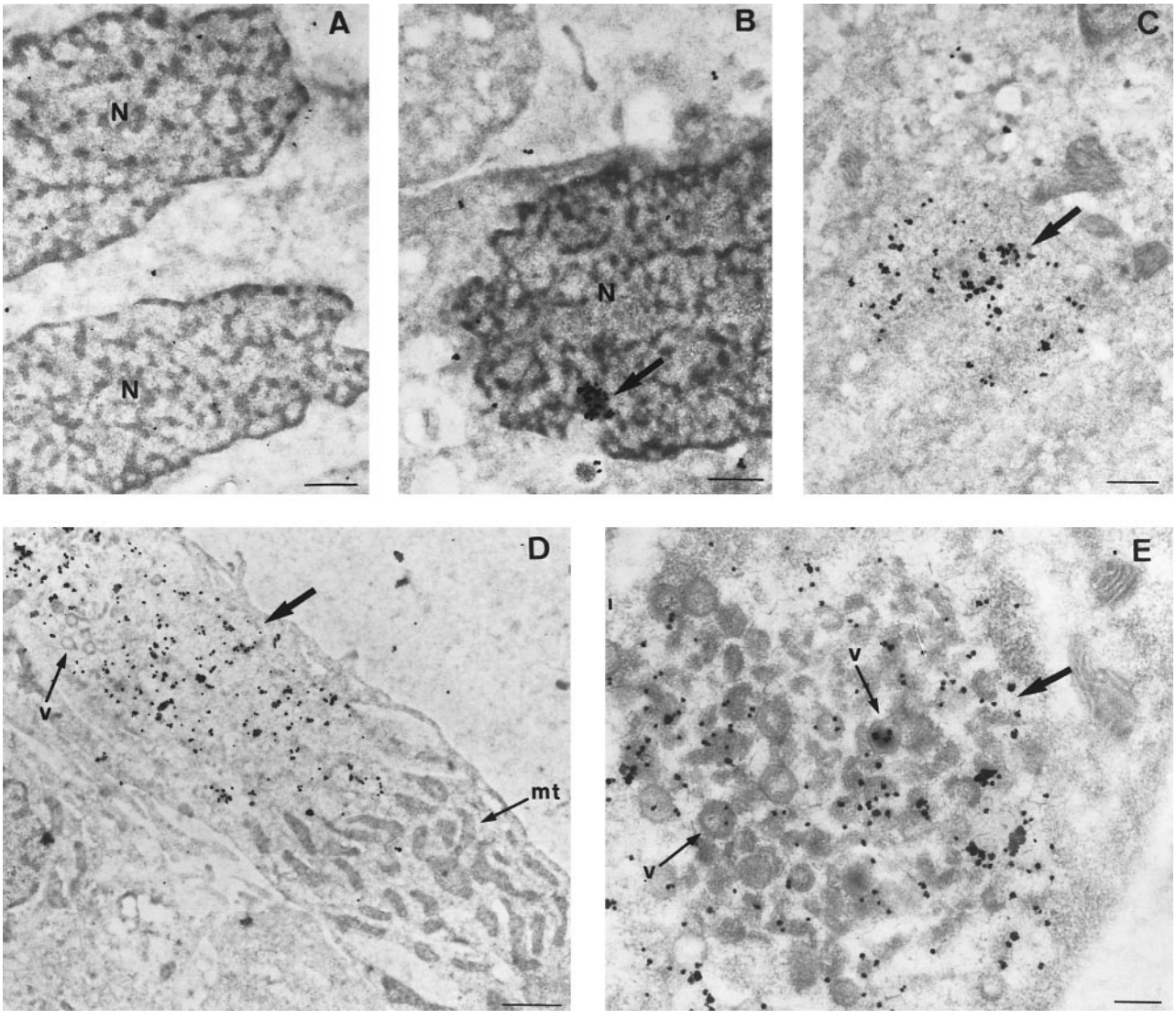

FIG. 1. Electron microscopic *in situ* hybridization of mock- and ASFV-infected Vero cells. Thin sections of mock- and ASFV-infected Vero cells were prepared at different times postinfection and then hybridized with a total virus DNA probe labeled with digoxigenin. The probe was detected with a sheep antidigoxigenin antibody and visualized with an anti-sheep antibody coupled to 10-nm gold particles. The size of the gold particles was increased by the procedure of silver enhancing. (A) Mock-infected cells at 8 h. N, nucleus. (B) Infected cells at 8 h. The hybridization signal indicated by an arrow is localized in the nucleus (N) in proximity to the nuclear membrane. (C) Infected cells at 12 h. The arrow points to a cytoplasmic signal. (D) Infected cells at 12 h. The hybridization signal (arrow) occupies a large cytoplasmic area that contains assembling virus particles (v). A large cluster of mitochondria (mt) can be seen in the periphery of this area. (E) Infected cells at 14 h. The hybridization signal (arrow) is localized in a virus assembly site. v, assembling virus particles. Bars in (A–C), 0.5 μm; in (D), 1 μm, and in (E), 0.2 μm.

phage system for the reasons mentioned above. Mock-infected and infected alveolar macrophages treated with 2 mM sodium butyrate were pulse-labeled for 30 min with [^{3}H]thymidine at different times postinfection, and at the end of the labeling period, the cells were subjected to a subcellular fractionation to separate the nucleus and the cytoplasm as indicated under Materials and Methods. After further purification of the nuclei, the nuclear and cytoplasmic fractions were lysed and centrifuged through a 5–20% linear alkaline sucrose gradient. Figure 4 shows the sedimentation profile of the nuclear and cytoplasmic DNA from mock-infected and infected cells labeled during the pulse at 4.5 h p.i. In the nuclear fraction from infected cells (Fig. 4A), small DNA fragments sedimenting at ~6–12S relative to λ DNA, which was carried in parallel gradients as a marker, were detected. These fragments may have an average size of ~2000 nucleotides. In the corresponding cytoplasmic fraction from infected cells (Fig. 4B), the main intermediates detected are of a slightly larger size (12–18S), but species sedimenting at ~34 and 46S were also found. These larger fragments were not detected in the nuclear fraction, indicating that the cytoplasmic material does not contaminate the purified nuclei preparation used in these studies. It should also be noticed that no genome-size DNA, which sediments at 65–93S (see below) or

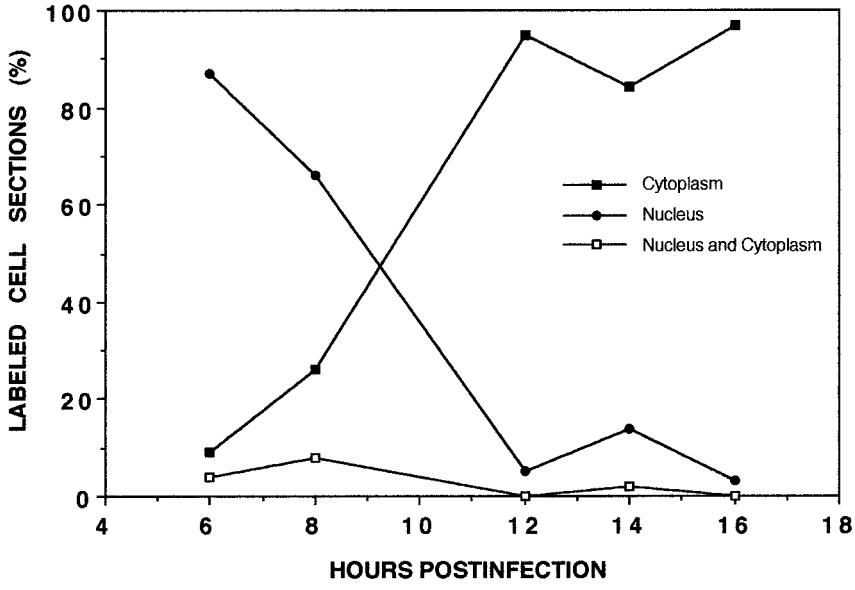

FIG. 2. Distribution of the hybridization signal in sections of ASFV-infected Vero cells. The percentage of cell sections with hybridization signal localized in the nucleus or in the cytoplasm was determined in infected Vero cells at the indicated times postinfection. The data are expressed as percentages from the total number of cell sections with label. Between 100 and 200 cell sections were counted at each point.

greater than unit-length molecules, were observed in this pulse.

Figure 5 shows the sedimentation profiles of the cytoplasmic fractions from mock-infected and infected macrophages labeled for 30 min at 12 and 17 h p.i. It should be mentioned that, as has been shown previously (García-Beato *et al.,* 1992b), the replication of the viral DNA in macrophages is exclusively cytoplasmic at these later times. As can be seen in the figure, DNA molecules with average sedimentation coefficients ranging from 37 to 49S were preferentially labeled at these times, but again no genome-size DNA molecules or concatemeric replicative intermediates were observed (Figs. 5A and 5B). We also examined the DNA species synthesized in the cytoplasm from infected cells in 30-min pulses at 15 and 19 h p.i., with similar results to those found at 12 and 17 h (not shown). Thus between 12 and 19 h p.i., the size of the predominant species synthesized is never larger than 37–49S. This size may correspond to about one-fourth to one-third of the genome.

To see whether the small cytoplasmic DNA fragments synthesized at 4.5 h p.i. are converted into the larger species detected at later times, pulse–chase experiments were performed. For this, the infected macrophages were pulse-labeled with [^{3}H]thymidine for 30 min at 4.5 h and then chased for 2 h in the presence of 2 mM unlabeled thymidine. These chase conditions were previously found to be completely efficient in preventing [^{3}H]thymidine incorporation and, on the other hand, do not inhibit viral DNA synthesis (García-Beato *et al.,* 1992b). As shown in Fig. 6A, the fragments of small size (~20S) synthesized during the pulse are converted into 30 and 40S species after the chase, indicating that the small cytoplasmic fragments are precursors of these larger species. Similar pulse–chase experiments to see whether the radioactivity in the DNA fragments detected in the nuclear fraction at 4.5 h p.i. was chased into the cytoplasmic intermediates were not feasible because of the radioactivity that is found in the cytoplasmic fraction even at this early time, probably due to the fact that the infection is not perfectly synchronized in the macrophage cell system.

In an attempt to detect the mature viral DNA, the infected macrophages were pulse-labeled with [^{3}H]thymidine for 30 min at a later time (12 h p.i.) and then chased for 1, 2, 4, and 8 h as before. Figure 6B shows that, after a chase of 1 h, the fragments of ~34–43S labeled during the pulse are converted into slightly larger intermediates. A minor peak corresponding to mature cross-linked DNA (93S) can also be observed. Similar results were obtained with a 2-h chase (not shown). After a 4-h chase, on the other hand, three fast-sedimenting DNA species were clearly resolved. These species have sedimentation coefficients of ~65, 80, and 93S, which are the coefficients, under alkaline conditions, of full-length viral DNA without cross-links, mature DNA with one cross-link, and mature DNA with two cross-links, respectively (Ortín *et al.,* 1979). These results indicate that the 34–43S intermediates synthesized during the pulse are precursors of the mature viral DNA. It should be mentioned, however, that the 65 and 80S species peaks could also correspond to molecules originated by nicking of cross-linked DNA at sites distinct from the termini. With longer chases (8 h), there was a considerable loss of radioactivity, probably due to the cell lysis which occurs at these late postinfection times, but it can

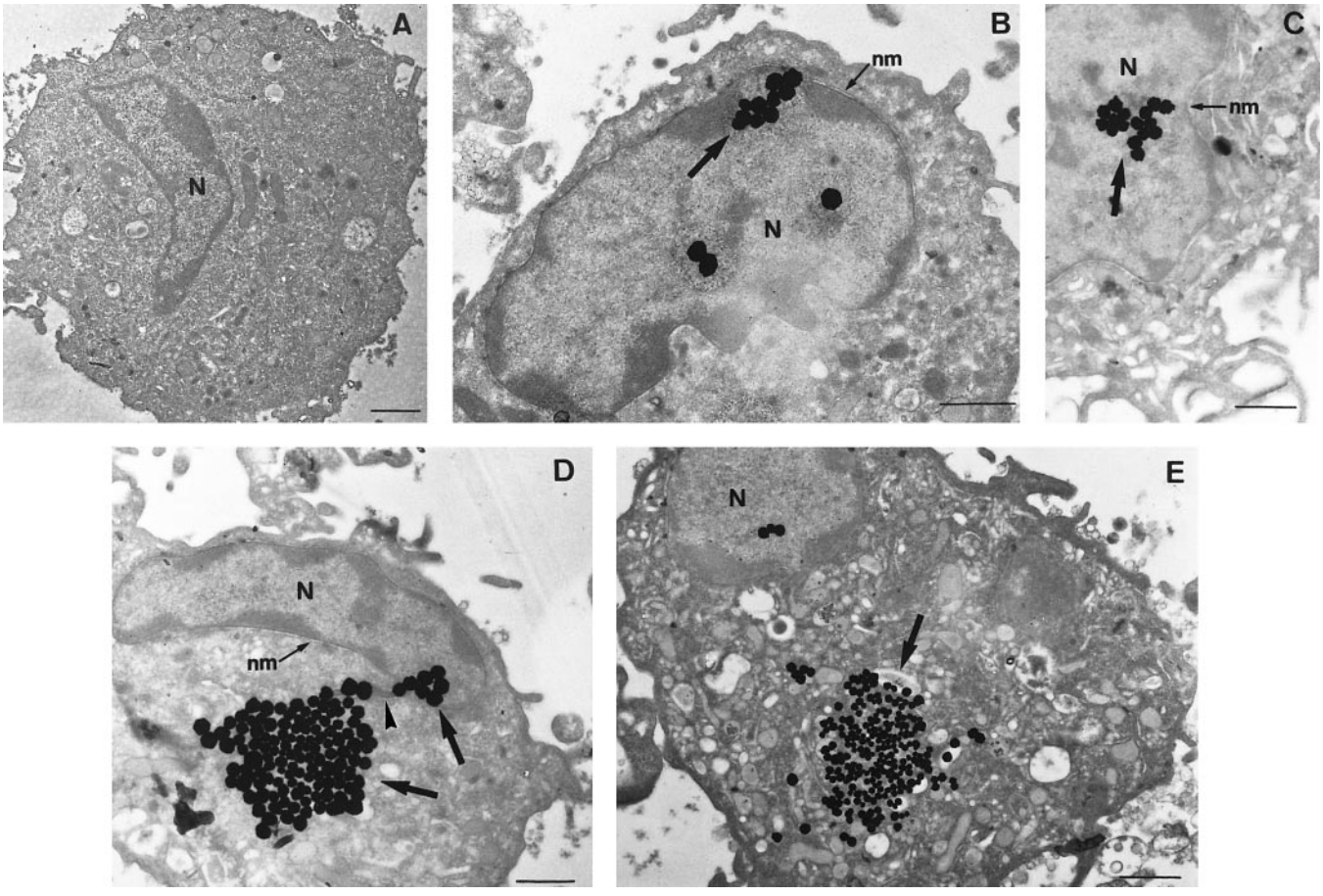

FIG. 3. Electron microscopic autoradiography of mock- and ASFV-infected alveolar macrophages. Porcine alveolar macrophages, preincubated with 2 mM sodium butyrate for 48 h to inhibit the synthesis of cellular DNA, were mock-infected or infected with ASFV and, at different times postinfection, they were pulse-labeled with [^{3}H]thymidine for 30 min. The cells were then processed for electron microscopic autoradiography as described under Materials and Methods. (A) Mock-infected cells at 4 h. N, nucleus. (B) Infected cells at 4 h. The arrow points to an autoradiographic signal localized in the nucleus (N) near the nuclear membrane (nm). (C) Infected cells at 5 h. The grains indicated by an arrow are localized in the nucleus. (D) Infected cells at 5 h. The autoradiographic signal (arrows) is found both in the nucleus and the cytoplasm. Notice that some of the grains follow a tract of electron dense material (arrowhead) that joins the nuclear and cytoplasmic foci. (E) Infected cells at 6 h. A large cytoplasmic signal is indicated by an arrow. Bars, 1 μm

be nevertheless seen that the predominant species is now the mature DNA with two cross-links (93S). These results suggest that the generation of the cross-links in the mature viral DNA is not a simultaneous but sequential process. On the other hand, no molecules greater than unit length were detected after any of the chase periods used in these experiments.

Detection of ASFV-specific DNA in nuclear and cytoplasmic fractions by dot-blot hybridization

To confirm that viral DNA is present in the nuclear fractions prepared from infected macrophages at early times postinfection, we hybridized these fractions as well as the corresponding cytoplasmic fractions to a virus-specific DNA probe. To this end, nuclear and cytoplasmic fractions, prepared from mock-infected and infected macrophages at different times postinfection as described before, were further processed as indicated under Materials and Methods. Aliquots of these fractions, equivalent to 100,000 cells, were applied to a nitrocellulose membrane and then hybridized to the ^{32}P-labeled *Hin*dIII-*Eco*RI subfragment of the ASFV DNA *Eco*RI D′ fragment (see Materials and Methods). As can be seen in Fig. 7, the hybridization signal corresponding to the nuclear fraction from infected cells at 4 h p.i. is similar to that of the mock-infected cells but increases considerably at 5 h. At a later time (6 h), a decrease in the intensity of the signal is observed. Figure 7 also shows the hybridization signal of the cytoplasmic fractions. A progressive increase in the signal corresponding to the infected cells is seen, with maximal intensity at 6 h p.i. These findings show that viral DNA is present in the nuclear fractions used in this study and are in line, on the other hand, with the present electron microscopic data, as well as with previous results (García-Beato *et al.,*

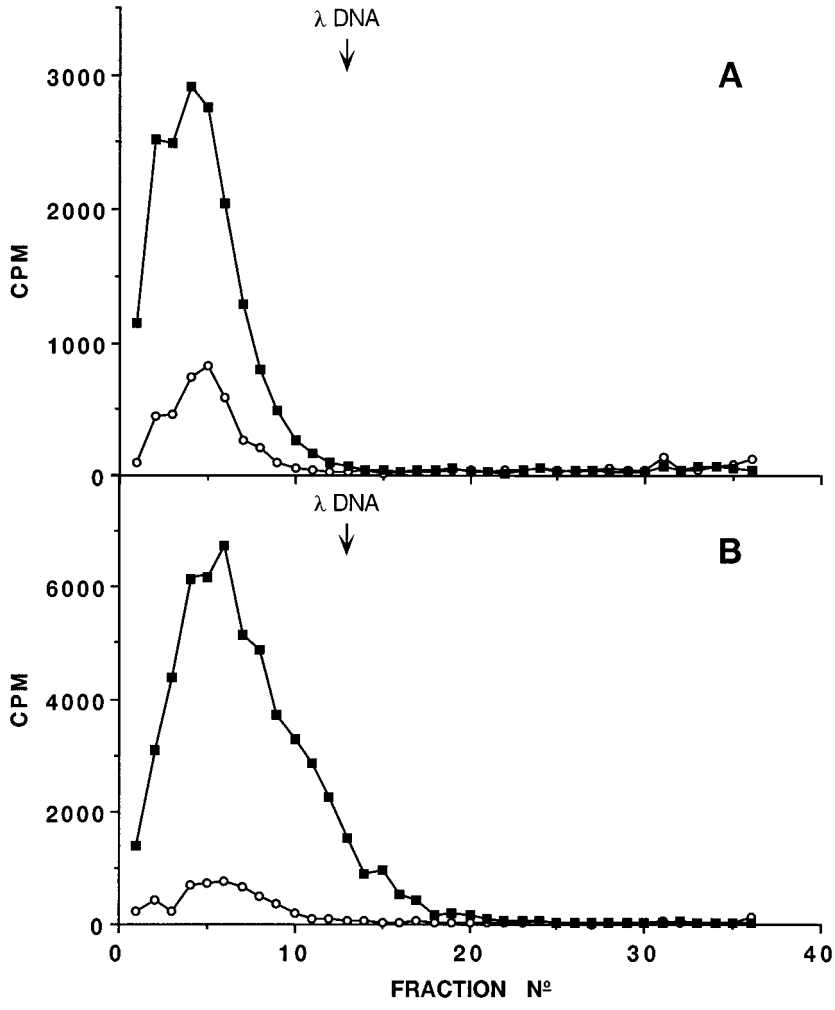

FIG. 4. Sedimentation analysis of replicating ASFV DNA labeled in the nucleus and cytoplasm of infected macrophages at an early time postinfection. Mock- and ASFV-infected alveolar macrophages, treated with sodium butyrate as described, were labeled with [^{3}H]thymidine for 30 min at 4.5 h p.i. Nuclear and cytoplasmic fractions were then prepared and centrifuged through a 5–20% alkaline sucrose gradient as indicated under Materials and Methods. One-milliliter fractions were then collected and the TCA precipitable radioactivity determined. The sedimentation position of λ DNA (40S), which was carried as a marker in a parallel gradient, is indicated. Sedimentation is from left to right. (A) Nuclear fractions from mock-infected cells (○) or ASFV-infected cells (■). (B) Cytoplasmic fractions from mock-infected (○) or infected cells (■).

1992b), that indicate that the nuclear stage in the replication of ASFV DNA precedes the cytoplasmic phase.

Analysis of replicating ASFV DNA by restriction enzyme treatment and pulsed-field electrophoresis

Although viral DNA molecules greater than unit length were not observed by sedimentation analysis in alkaline sucrose gradients, previous work has shown, as mentioned in the Introduction, that head-to-head and tail-to-tail linked molecules can be detected in ASFV-infected Vero cells after treatment of replicating viral DNA with restriction enzymes. These molecules could be derived from replicative concatemeric intermediates, although they have been found at a very late time postinfection (48 h) (González *et al.,* 1986). To confirm these findings in macrophages, as well as to examine in more detail the times postinfection at which these molecules are generated, mock- and ASFV-infected macrophages were collected at different times postinfection, and the total cell DNA was extracted and then subjected to treatment with *Hin*dIII as described under Materials and Methods. After digestion, the fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose, and then hybridized to the ^{32}P-labeled probe used in the dot-blot hybridization. This probe contains the virus terminal inverted repetitions and hybridizes with the *Hin*dIII terminal fragments from both DNA ends. As shown in Fig. 8, two bands of 2.3 and 9 kb were detected in the infected cells at 8 h p.i. and later. These bands correspond to the terminal fragments that are produced when the ASFV DNA isolated from virions is digested with *Hin*dIII (González *et al.,* 1986). In addition to these bands, two other bands of 4 and 18 kb, about twice the size of each

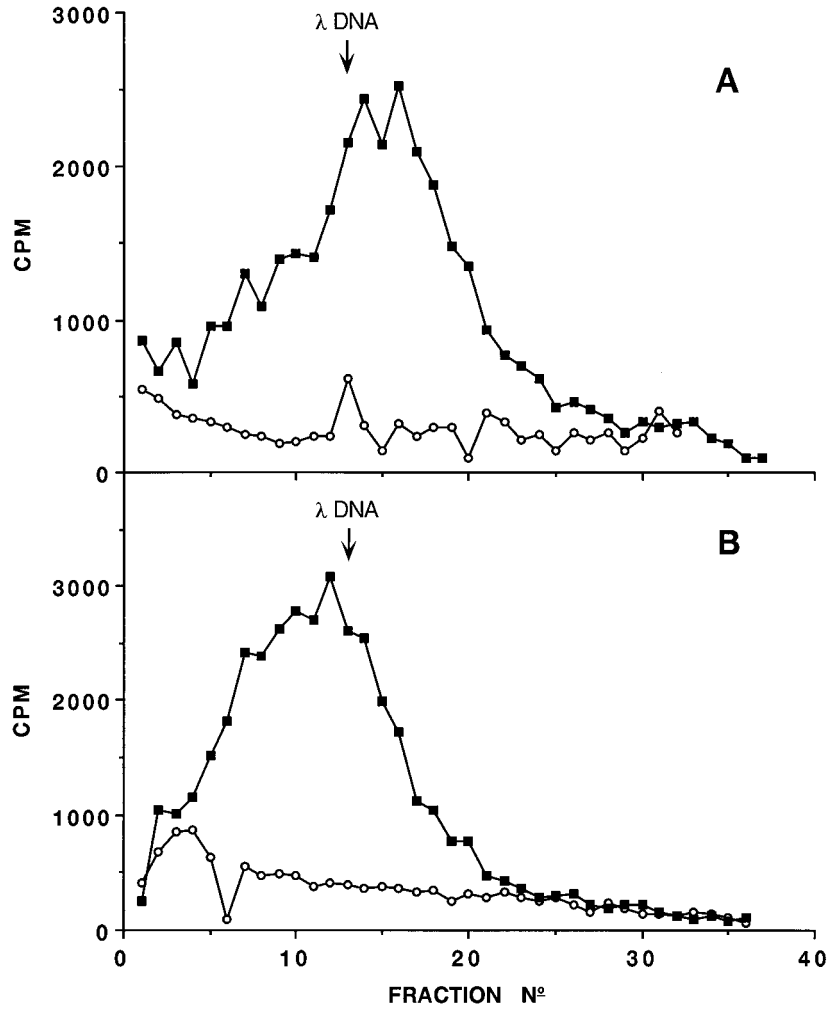

FIG. 5. Sedimentation analysis of cytoplasmic ASFV DNA synthesized in infected macrophages at intermediate and late times postinfection. Mock-infected (○) and infected (■) macrophages were labeled with [^{3}H]thymidine for 30 min at 12 (A) and 17 (B) h p.i. and the cytoplasmic fractions were then processed as indicated in the legend to Fig. 4. Sedimentation is from left to right. The sedimentation position of λ DNA is indicated.

terminal *Hin*dIII fragment, were found. These dimeric terminal fragments were also detected at 8 h p.i. and later but are in a lower proportion than the monomeric terminal fragments. It should be noticed, however, that the left dimeric fragments are in larger amounts than the right ones. Thus the dimeric ASFV terminal fragments might be derived from viral concatemeric replicative intermediates, which are predominantly head-to-head linked.

To see whether viral DNA molecules greater than unit length are indeed generated in ASFV-infected macrophages, we have analyzed the replicating viral DNA by pulsed-field electrophoresis. To this end, the mock-infected and infected macrophages were processed at different times postinfection as indicated under Materials and Methods. The samples were then subjected to pulsed-field electrophoresis, and after transfer to a nitrocellulose membrane, the viral DNA was detected by hybridization to ^{32}P-labeled total ASFV DNA. As shown in Fig. 9, two bands are detected. One of them has the size of genomic DNA (170 kb), being first observed at 8 h p.i., and greatly increasing in amount at later times postinfection. The second band migrates more slowly and is found in a minor proportion relative to genomic DNA. This band, which is weakly detected at 12 h p.i., but is more intense at 17 h, has a size of 350 kb, thus being a linear dimeric viral concatemer. No oligomeric concatemers, up to hexamers, were found. As suggested by the restriction data, this dimeric concatemer is predominantly head-to-head linked. On the other hand, a high-molecular-weight material is retained at the origin of the gel. This material, which increases as the infection progresses, could represent a circular form of the dimeric concatemer or, alternatively, linear multimeric concatemers greater than hexamers. Attempts to distinguish between these two possibilities, by varying the pulse conditions in the electrophoresis, have been unsuccessful, although it is unlikely that the retained material corresponds to multimeric concatemers, as no trimers to hexamers have been detected.

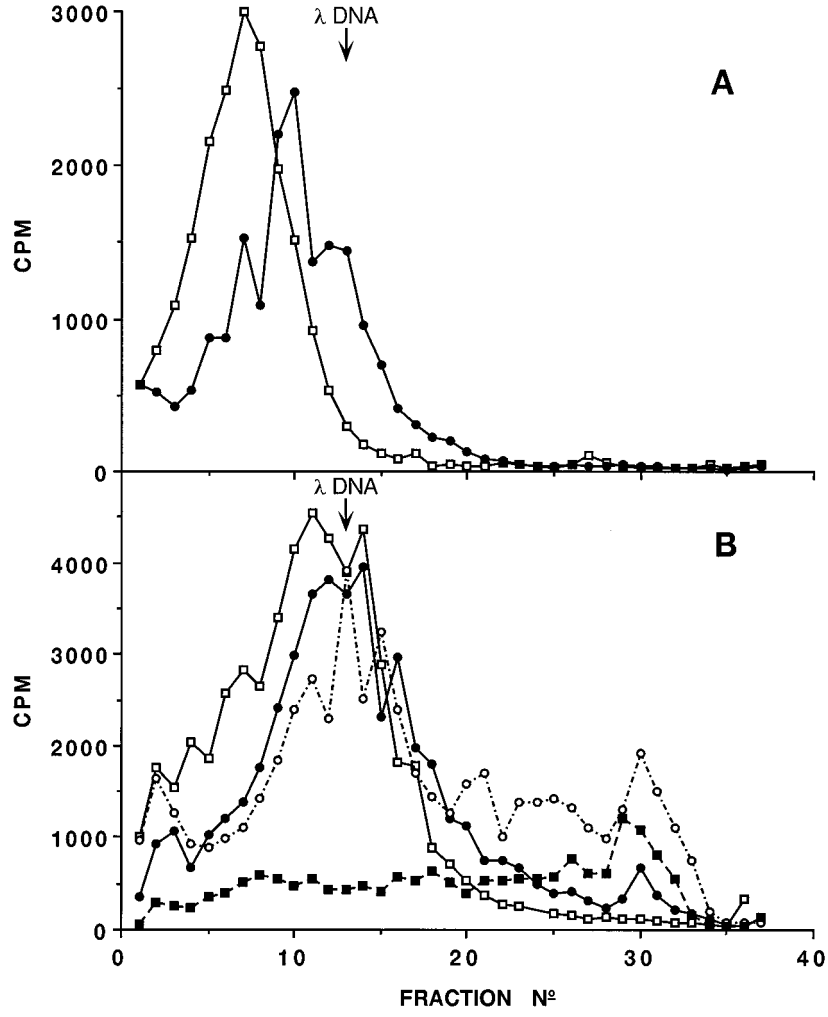

FIG. 6. Sedimentation analysis of replicating ASFV DNA synthesized during pulse chases. (A) Infected macrophages were pulse-labeled with [^{3}H]thymidine for 30 min at 4.5 h p.i. and then chased for 2 h in the presence of 2 mM unlabeled thymidine. At the end of the pulse and the chase, the cytoplasmic fractions were analyzed as described in the legend to Fig. 4. □, 30-min pulse; ●, 2-h chase. (B) Infected macrophages were pulse-labeled with [^{3}H]thymidine for 30 min at 12 h p.i. and then chased for different time periods as before. □, 30-min pulse; ●, 1-h chase; ○, 4-h chase; ■, 8-h chase. Sedimentation is from left to right. The sedimentation position of λ DNA is indicated in (A) and (B).

DISCUSSION

This study on the ultrastructural localization of ASFV DNA in infected cells provides further evidence of the existence of an initial nuclear stage in the replication of the virus DNA. Examination of infected Vero cells by electron microscopic *in situ* hybridization shows the presence of virus DNA foci in the nucleus in a high percentage (90%) of the cells at early times in the replication of the viral DNA. At later times, the virus DNA was found exclusively in the cytoplasm, in agreement with previous data obtained by light microscopy (García-Beato *et al.,* 1992b). Virus morphogenesis takes place in the cytoplasmic DNA containing areas as the assembling virus particles were found to colocalize with the virus DNA. On the other hand, electron microscopic autoradiography of ASFV-infected macrophages shows that the nucleus of the host cell is also a site of viral DNA replication. The high percentage of Vero cells with virus DNA signal in the nucleus strongly supports the biological relevance of the nuclear stage in the replication of ASFV DNA. It is noteworthy, on the other hand, that the nuclear foci are always localized in proximity to the nuclear membrane, which suggests the existence of a specific mechanism that anchors the virus DNA to this nuclear region. An attractive hypothesis is that the cellular topoisomerase II would play a role in this anchorage by binding both to the centromeric satellite DNA of the heterochromatin (Käs and Laemmli, 1992), which is localized near the nuclear membrane, and to the satellite-like sequences that have been described in ASFV DNA (Almazán *et al.,* 1995). A similar role could be proposed for the viral topoisomerase II (García-Beato *et al.,* 1992a), which is expressed at early times postinfection (A. Alejo and M. L. Salas, unpublished results) and

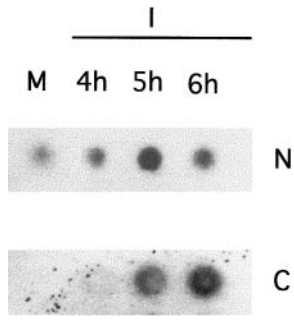

FIG. 7. Detection of ASFV DNA in nuclear and cytoplasmic fractions from infected macrophages by dot-blot hybridization. Nuclear and cytoplasmic fractions were prepared from mock-infected and infected macrophages at the indicated times and processed as described under Materials and Methods. Aliquots of these fractions, equivalent to 10,0000 cells, were applied to a nitrocellulose membrane and hybridized to the ^{32}P-labeled probe described under Materials and Methods. N, nuclear fractions; C, cytoplasmic fractions; M, mock-infected cells; and I, infected cells.

might also interact with both the cellular and viral satellite sequences.

The analysis of the ASFV replicative intermediates that are synthesized in the nucleus and cytoplasm of infected macrophages has provided, on the other hand, information on the events in the synthesis of the viral DNA that occur in the nucleus and cytoplasm as well as on the molecular mechanism of genome replication. The results obtained by alkaline sucrose sedimentation analysis indicate that, in 30-min pulses, DNA fragments of small size (~6–12S), with an average length of ~2000 nucleotides, are synthesized in the nucleus at early times in the replication of the viral DNA. At these early times, slightly larger intermediates (12–18S) are preferentially labeled in the cytoplasm, while at later times, between 12 and 19 h p.i., the cytoplasmic intermediates sediment at ~37–49S, with a length corresponding from one-fourth to one-third of the genome. These intermediates are precursors of mature viral DNA, as demonstrated in pulse–chase experiments. Three classes of genome-size molecules were detected after the chases: full-length DNA without cross-links and mature DNA with one or two cross-links. The introduction of the cross-links in the mature ASFV DNA appears to be a sequential process, as the molecules without and with only one cross-link detected at the end of a 4-h chase are converted into the species with two cross-links after a longer chase (8 h). On the other hand, the finding that in 30-min pulses between 12 and 19 h p.i. the predominant molecules labeled are never larger than one-fourth to one-third of the genome size may suggest that the generation of the mature size viral DNA involves ligation of the 37–49S intermediates. These findings suggest that the replication of ASFV DNA proceeds through the synthesis of small (6–18S) fragments, which are then converted into intermediate size molecules (37–49S) and finally into the mature cross-linked DNA. In relation to this, it is interesting to mention that earlier work on the replication of vaccinia virus DNA, using alkaline sucrose gradient centrifugation to analyze the replicating viral DNA, indicated that the process of vaccinia virus DNA replication involves the synthesis of small DNA fragments (8–12S), which are then converted into 30–50S intermediates and

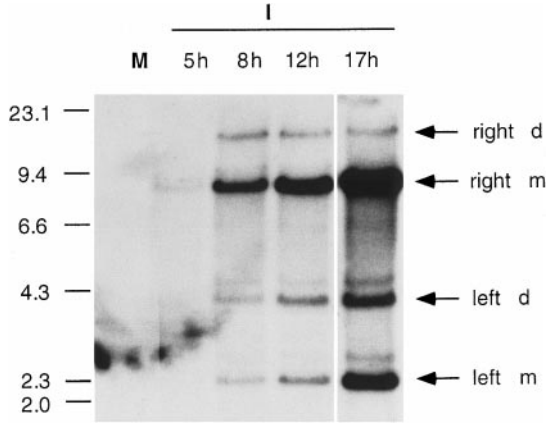

FIG. 8. Restriction enzyme analysis of ASFV DNA isolated from infected macrophages. Total cell DNA was extracted from mock-infected and infected macrophages at the indicated times postinfection, digested with *Hin*dIII, and processed as described under Materials and Methods. The fragments were detected by hybridization with the ^{32}P-labeled terminal *Hin*dIII-*Eco*RI subfragment of ASFV DNA *Eco*RI D′ fragment. M, mock-infected cells; I, infected cells; Left m, left monomeric terminal fragment (2.3 kb) from ASFV DNA; left d, left dimeric terminal fragment (4.0 kb); right m, right monomeric (9 kb) terminal fragment; and right d, right dimeric (18 kb) terminal fragment. Size markers in kb are indicated on the left.

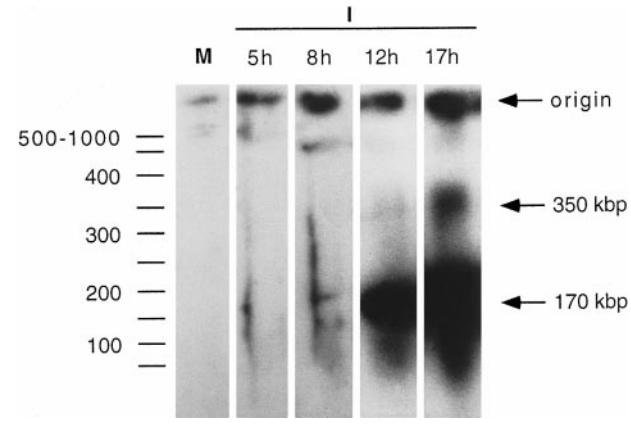

FIG. 9. Detection of ASFV DNA in infected macrophages by pulsed-field electrophoresis. Whole cell extracts from mock-infected and infected macrophages were prepared at the indicated times postinfection and processed as described under Materials and Methods. The DNA was separated by pulsed-field electrophoresis, and after transfer to nitrocellulose membranes, the DNA was hybridized with a ^{32}P-labeled total ASFV DNA probe as indicated under Materials and Methods. M, mock-infected cells; I, infected cells. The position of genomic (170 kb) and dimeric (350 kb) ASFV DNA is indicated. Concatemers of λ phage DNA from Biorad, used as size markers, are indicated in kb on the left.

finally into full-length DNA (Esteban and Holowczak, 1977; Esteban *et al.,* 1977; Pogo and O'Shea, 1978; Pogo *et al.,* 1981). As a postreplicative event, the viral DNA acquires the cross-links and is covalently closed (Esteban and Holowczak, 1977; Pogo and O'Shea, 1978). These findings on vaccinia virus DNA synthesis are very similar to those described here for the replication of ASFV DNA. Even the size of the different intermediates detected in both cases is remarkably similar. This suggest that vaccinia virus and ASFV share common aspects in the mechanism of genome replication, as could be expected given the structural similarities between the DNA of these two viruses.

In addition to the data obtained by sedimentation analysis on the replication of ASFV DNA, pulsed-field electrophoresis of replicating viral DNA has allowed to detect, at intermediate and late times in the synthesis of ASFV DNA, a linear dimeric concatemer, which, as suggested by restriction enzyme analysis, may be predominantly head-to-head linked. Head-to-head and tail-to-tail linked concatemers have also been found in vaccinia virus-infected cells (Baroudy *et al.,* 1982b; Moyer and Graves, 1981). These concatemers are intermediates in the replication of the viral DNA and are resolved to generate the mature vaccinia virus DNA (DeLange, 1989; Merchlinsky and Moss, 1989). Likewise, the dimeric concatemer found in the ASFV-infected cells could be a precursor of genomic DNA. The finding that the ASFV concatemer is detected in a considerably lower proportion than the genomic DNA at the different times postinfection examined could be due to a rapid resolution of the concatemer. On the other hand, and although a self-priming mechanism has been proposed for the generation of the vaccinia virus concatemers (Moyer and Graves, 1981), it is unlikely that such a mechanism may operate in the case of ASFV DNA as no radioactivity associated to genome-size DNA has been detected at the earliest time postinfection analyzed. Also the small DNA fragments synthesized at this time do not support the self-priming mechanism. It should be mentioned, however, that the possibility that these fragments could be originated by breakage of long DNA strands synthesized by a continuous mechanism can not be completely ruled out at present. The way in which the dimeric ASFV concatemer could be generated will be discussed below.

Altogether, our findings support a mechanism of ASFV DNA replication similar to that postulated in the *de novo* start model of Baroudy *et al.* (1982b) for the replication of vaccinia virus DNA. As in this model, we propose that the replication of ASFV DNA would be initiated at both ends of the genome, with the synthesis of leading and lagging strands, possibly carried out by the virus-encoded replicative DNA polymerase (Rodríguez *et al.,* 1993). The small DNA fragments that we detect at early times in the synthesis of ASFV DNA are consistent with this mechanism. The synthesis of leading and lagging strands may involve the use of RNA fragments as primers, although no gene with homology to primases has been identified in the ASFV genome (Yáñez *et al.,* 1995). A possible explanation for the requirement of the nucleus in ASFV DNA replication would thus be the need for the primase subunits of the cellular DNA polymerase α (Copeland and Wang, 1993). Complete replication of the genome by this mechanism would originate a two-unit concatemer with dimeric ends. The ASFV dimeric concatemer detected at intermediate and late times as well as the dimeric head-to-head and tail-to-tail linked terminal fragments found at these times are also in line with this proposal. Separation of the strands of the dimeric concatemer would be accomplished, as postulated by Baroudy *et al.* (1982b), by site-specific nicking within the inverted terminal repetition and rearrangement, which would be followed by ligation. While this latter step could be performed by the virus-encoded ligase (Yáñez and Viñuela, 1993), the enzyme that might catalyze the nicking reaction is unknown at present. Although the ASFV concatemer detected is a linear form and the concatemer generated in the Baroudy *et al.* model would be a circular structure, nicking at one of the ends of the ASFV concatemer could explain this difference. The finding that the left dimeric fragments from replicating ASFV DNA are in larger amounts than the right ones suggests that nicking occurs first at the right end (tail) of the concatemer with the generation of a linear head-to-head linked molecule. Finally, the proposed nicking of the concatemer could also explain the inability to detect the ASFV concatemer in alkaline sucrose gradients, as nicking at one or both ends of the concatemer would originate molecules that would be detected in the gradients as genome-size DNA with one cross-link or genome-size single-stranded DNA without cross-links, respectively.

MATERIALS AND METHODS

Cells and viruses

The ASFV strain BA71 has been described before (Enjuanes *et al.,* 1976). BA71V is the isolate BA71 adapted to grow in Vero cells (Enjuanes *et al.,* 1976). Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. Pig alveolar macrophages were prepared by broncoalveolar lavage as previously reported (Carrascosa *et al.,* 1982). They were cultured in DMEM containing 10% swine serum.

Electron microscopic *in situ* hybridization of ASFV-infected Vero cells

Vero cells were grown in plastic dishes and, when the cultures reached the density of 80,000 cells per cm^2, they were mock-infected or infected with ASFV strain BA71V at 5 PFU per cell. At different times postinfection, they

were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde, dehydrated in ethanol, and embedded in Lowicryl K4M. Thin sections (80 nm) of these cells were treated with 2 μg per ml of proteinase K in 10 mM Tris–HCl, pH 7.4 for 15 min at 37°C, digested with 100 μg per ml of RNase A in 2× SSC for 1 h at 37°C, and finally incubated with 70% deionized formamide in 2× SSC for 2 min at 70°C to denature the intracellular DNA. Prehybridization of the samples was performed in the presence of 50% deionized formamide, 2× SSC, 10 mM Tris–HCl, pH 7.5 and 1 mM EDTA for 1 h at 37°C. For hybridization, the probe was total ASFV DNA isolated from virions purified as reported (Carrascosa *et al.,* 1985) and digested with *Hin*dIII. The probe was labeled with digoxigenin-11-dUTP (Boehringer Mannheim Biochemica) as described in the applications manual from Boehringer and was denatured by heating at 100°C for 5 min. The hybridization solution contained 50% deionized formamide, 2× SSC, 10% dextrane sulfate, 15× Denhardt's, 100 μg/ml calf liver tRNA, 100 μg/ml salmon testis DNA, and 5 μg/ml of the probe. Ten microliters of this solution were applied per grid, and the samples were hybridized overnight at 37°C. After hybridization, the grids were blocked with 2% dry milk powder in PBS for 15 min at room temperature. To detect the probe, the samples were first incubated with a 1:100 dilution of a sheep antidigoxigenin antibody in PBS containing 2% dry milk powder and then with an anti-sheep antibody coupled to 10-nm gold particles. The samples were then treated with a "silver-enhancing" solution (Biocell Research Laboratories) for 3 min at room temperature to increase the size of the gold grains. Finally, the sections were stained with 2% uranyl acetate and 0.2% lead citrate. The samples were examined under a Jeol 1010 electron microscope.

Detection of newly synthesized ASFV DNA in infected macrophages by electron microscopic autoradiography

Alveolar macrophages were seeded in plastic dishes at a density of 2×10^6 cells/ml, allowed to adhere to the dishes for 48 h and then incubated with 2 mM sodium butyrate for 24 h. At the end of this period, the cells were mock-infected or infected with the ASFV isolate BA71 at a m.o.i. of 20 hemadsorption units (HADU) per cell. At different times postinfection, the cells were pulse-labeled for 30 min with 30 μCi/ml [^{3}H]thymidine (45 Ci/mmol; Amersham), and then fixed with 2% glutaraldehyde and 2% tannic acid, dehydrated in ethanol, and stained with 2% uranyl acetate. After embedding in Epon, thin sections of 80 nm were cut, placed in slides, and dipped in Ilford L-4 emulsion and exposed at 4°C in the dark for 2 months. After exposure, the samples were developed in Phenidon developer, and finally stained with 2% lead citrate.

Sedimentation analysis of replicating ASFV DNA in alkaline sucrose gradients

For pulse-labeling experiments, the macrophages were treated with sodium butyrate as described above and mock-infected or infected with ASFV BA71 at a m.o.i. of 5 HADUs per cell. At different times postinfection, the cells were labeled for 30 min with 30 μCi/ml [^{3}H]thymidine (45 Ci/mmol), and, at the end of the pulse period, nuclear and cytoplasmic fractions were obtained, and the nuclei were purified by a modification of the Berkowitz *et al.* (1969) procedure, as described by Goorha *et al.* (1978). After purification, the nuclei were suspended in 50 mM HEPES, pH 8, 10 mM EDTA, and 0.22 M sucrose, layered on top of a 1 ml lysis layer (0.5 M NaOH and 10 mM EDTA) placed above the 5–20% alkaline sucrose gradient (Goorha *et al.,* 1978) and allowed to stand overnight at 4°C. The cytoplasmic fractions were adjusted to 50 mM HEPES, pH 8, 10 mM EDTA, and 0.22 M sucrose and were processed similarly. The gradients were then centrifuged in an SW27 rotor for 18 h at 15,000 rpm. After centrifugation, 1-ml fractions were collected, precipitated with 10% TCA and the radioactivity counted. For pulse–chase experiments, infected macrophages were pulse-labeled for 30 min at 4.5 or 12 h p.i. with [^{3}H]thymidine as before, and at the end of the labeling period, the medium was removed and the cultures were washed three times with PBS. Fresh medium containing 2 mM thymidine was then added, and the cells were further incubated for 1, 2, 4, and 8 h. The cells were then processed as just described.

Dot-blot hybridization of nuclear and cytoplasmic fractions using an ASFV-specific DNA probe

Sodium butyrate-treated alveolar macrophages were mock-infected or infected with ASFV and, at different times postinfection, fractionated as described. The nuclear and cytoplasmic fractions were first treated with 100 μg/ml proteinase K for 1 h at 37°C and then boiled for 5 min in the presence of 0.3 M NaOH. The samples were neutralized with 1 M Tris–HCl, pH 7.5 and 1 M HCl and adjusted to 6× SSC. Aliquots of the nuclear and cytoplasmic fractions corresponding to 100,000 cells were applied to a nitrocellulose membrane and hybridized to a virus-specific DNA probe. To prepare the probe, plasmid p5RD′ (Ley *et al.,* 1984) containing fragment *Eco*RI D′ of ASFV DNA was digested with *Eco*RI and *Hin*dIII, and the ASFV 8.8-kb terminal *Hin*dIII-*Eco*RI subfragment was labeled by random priming using [α-^{32}P]dCTP. Prehybridization and hybridization were performed as described elsewhere (Sambrook *et al.,* 1989).

Restriction enzyme analysis of replicating ASFV DNA

Sodium butyrate-treated mock- and ASFV-infected alveolar macrophages were collected at different times

postinfection, and the total cell DNA was extracted, digested with *Hin*dIII, and subjected to electrophoresis in a 0.5% agarose gel. After transfer to a nitrocellulose membrane, the DNA was hybridized with the ^{32}P-labeled *Hin*dIII-*Eco*RI subfragment of ASFV DNA described above.

Analysis of replicating ASFV DNA by pulsed-field electrophoresis

Alveolar macrophages treated with sodium butyrate were mock- or ASFV-infected and collected at different times postinfection. The cells were resuspended in a buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA (NB buffer), and 400 μg/ml of proteinase K, and mixed with an equal volume of 1.5% low-melting-point agarose in NB buffer at 50°C. The mixture was allowed to solidify and was then incubated overnight at 37°C in sarkosyl lysis buffer (10 mM Tris–HCl, pH 9.5, 10 mM NaCl, 25 mM EDTA, 1% sarkosyl) containing 200 μg/ml proteinase K. After washing three times with 10 mM Tris–HCl, pH 7.5 and 1 mM EDTA, the blocks were inserted into wells, and electrophoresis was run in 1% agarose gels for 18 h at 11°C and 200 V, with pulse times of 30 s in a Chef-DRII electrophoresis unit (Biorad). After electrophoresis, the DNA was transferred to a nitrocellulose membrane and hybridized with total ASFV DNA isolated from virions and labeled by random priming with [α-^{32}P]dCTP.

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