Requirement of Cell Nucleus for African Swine Fever Virus Replication in Vero Cells

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African swine fever (ASF) is a highly lethal disease of pigs caused by an enveloped icosahedral virus (for a review see reference 13), whose genome is a double-stranded DNA molecule with a molecular weight of ~100 × 10^6 (1, 6, 20).

The replication of the virus takes place in the cytoplasm of the infected cell (2), and, therefore, it has been tentatively classified as an icosahedral cytoplasmic deoxyvirus (7, 14, 16).

The availability of methods to prepare mass cultures of enucleated cells by cytochalasin treatment (3, 8, 21) has allowed the investigation of the role of the cell nucleus in the development of viruses which grow in the cytoplasm of infected cells (9, 10, 18, 21). According to this type of analysis, the viruses studied could be classified into two groups: those that do not require the cell nucleus at all, such as poliovirus, Sindbis virus, and vesicular stomatitis virus, and those that require nuclear DNA replication and transcription, such as RNA tumor viruses, or just DNA transcription, such as myxoviruses.

By using a similar type of approach, we present in this report experiments designed to ascertain the possible roles of the cell nucleus in the development of ASF virus in Vero cells.

MATERIALS AND METHODS

Cells and viruses. The origin of the ASF virus strain used in this laboratory as well as its adaptation to grow on Vero cells has been previously described (5).

The Vero cell line (CCL81) was obtained from the American Type Culture Collection, and the BHK-21 cell line and Sindbis virus seeds were gifts from D. T. Brown, Institute of Genetics, Cologne.

Chemicals. Calf serum was purchased from Grand Island Biological Co., trypsin from Difco Laboratories, cytochalasin B from Calbiochem, agarose (electrophoretic grade) from Sigma Chemical Co., and Nonidet P-40 (NP-40) from BDH. Plastic labware for tissue culture was purchased from Falcon Plastics, and nitrocellulose filters for DNA-DNA hybridization (HAWP, 0.45-µm pore size) were from Millipore Corp. (methyl-3H)thymidine (specific activity, 19 Ci/mmole) was bought from the Radiochemical Centre, Amersham, England.

Solutions. NP-40 solution for cell fractionation contained 0.6% NP-40-0.15 NaCl-0.0015 M MgCl2-0.01 M Tris-hydrochloride, pH 8.5. The composition of phosphate-buffered saline (Ca2+- and Mg2+-free) has been described previously (4).

Plaque assay. The conditions for plaque assay of ASF virus on Vero cells have been described (5). Sindbis virus was assayed on BHK-21 cells as described by Pfefferkorn and Hunter (19).

Enucleation and infection conditions. The enucleation procedure was as described by Prescott et al. (21). Briefly, Vero cells grown on plastic cover slips (24-mm diameter) were enucleated by treatment with 10 µg of cytochalasin B per ml in Dulbecco medium supplemented with 10% calf serum and centrifugation for 50 min at 3,000 × g in an SS34 rotor and Sorvall centrifuge, prewarmed to 35 to 37°C. Control cells were treated in the same way but were not centrifuged.

After each centrifugation, cell losses and enucleation efficiency were controlled. Cell losses, determined by counting cells in control cultures, ranged between 10 and 20%. The enucleation efficiency was determined by counting cells remaining nucleated in control cultures, after fixation and staining with aceto-orcein. Routine values were between 90 and 98%.

After a recovery period of 1 h at 37°C, the cells were infected with ASF virus at 20 PFU/cell or Sindbis virus at 200 PFU/cell or mock infected. The inoculum was allowed to adsorb for 2 h at 37°C, and the cells were washed twice with fresh medium and further incubated at 37°C. In some experiments adsorption and incubation were performed at 32°C.

Labeling conditions and cell fractionation. To study the kinetics of viral DNA synthesis, ASF virus-infected or mock-infected cells, either normal or enucleated, were pulse labeled for 3 h with [methyl-3H]thymidine at 20 µCi/ml in Dulbecco medium sup-
plemented with 2% calf serum. After the pulse period, the cells were washed with cold phosphate-buffered saline and lysed in isotonic buffer containing 0.6% NP-40 for 10 min at 0°C. The lysate was shaken several times in a Vortex mixer, and the nuclei were removed by centrifugation for 3 min at 1,000 × g and 4°C. The cytoplasmic fraction was precipitated with trichloroacetic acid, and the precipitate was counted in a toluene-based scintillator.

DNA-DNA hybridization. Cold virus DNA and labeled cytoplasmic DNA were extracted by the procedure of Gross-Bellard et al. (12). The purification of ASF virus for extraction of DNA was performed by described methods (6), involving fractionation of infected cells into nuclear and cytoplasmic extracts and equilibrium centrifugation of the latter onto sucrose gradients. The DNA extracted from virus purified in this way sediments as a single component, with a sedimentation value of 58S in neutral sucrose gradients (6; L. Enjuanes, personal communication).

The hybridization of pulse-labeled cytoplasmic DNA with ASF virus DNA in nitrocellulose filters was performed by the method of Green et al. (11).

RESULTS

Growth of ASF in enucleated cells. In all the experiments to be described here, the capacity of the enucleated cells to support virus growth was tested by infection of replicate cover slips with Sindbis virus, which is reported to replicate satisfactorily in enucleated cells (15). A single stock of Sindbis virus, grown on BHK-21 cells, was used. The yield of Sindbis virus in enucleated Vero cells was about 50% of that obtained in control, cytochalasin B-treated, uncentrifuged cells.

Under these conditions, replicate cover slips were infected with ASF virus after enucleation, and the growth kinetics were followed by plaque assay. Figure 1 shows that enucleated cells do not support the replication of ASF virus at values higher than those to be expected for the background of cells remaining nucleated, whereas the replication of Sindbis virus in enucleated cells was normal although somewhat delayed with respect to the control nucleated cells.

Kinetics of viral DNA synthesis in enucleated Vero cells. To ascertain the possible steps in the ASF virus growth cycle affected by the absence of nuclei, experiments were performed in which enucleated Vero cells were infected and pulse labeled at different times after infection with [methyl-3H]thymidine. Figure 2 presents the kinetics of the incorporation of the precursor into trichloroacetic acid-insoluble material in the cytoplasm of control or enucleated cells. The results suggest that enucleated cells are unable to support ASF virus DNA replication, the residual DNA synthesis being probably due to the incorporation in the cells remaining nucleated in the cultures.

Furthermore, the nature of the pulse-labeled, cytoplasmic DNA synthesized in control infected cultures was determined by hybridization with ASF virus DNA. According to this criterion most of the newly synthesized DNA was characterized as virus specific (Table 1).

**Enucleation p.i. of Vero cells by ASF virus.** To examine further the role of the cell nucleus in the infection of Vero cells by ASF virus, infected cultures were subjected to the enucleation procedure at different times postinfection (p.i.), up to 10 h p.i. The enucleated cultures were incubated further, and the yield of the virus progeny was measured at 50 h p.i. Figure 3 shows the results of such an experiment. As a control, Sindbis virus-infected Vero cells were enucleated at 2 h p.i., and the growth kinetics of the virus were determined by plaque assay (Fig. 3, insert). The results, expressed as the percentage of the yield in control cells that were treated with cytochalasin B at 2, 6, or 10 h p.i. indicate that there is no detectable growth of ASF virus in enucleated cells, even when enucleation was performed up to 10 h p.i.
Fig. 2. Vero cells were grown, enucleated, and infected with ASF virus as described in the legend to Fig. 1. At the times after infection indicated in the figure, control or enucleated cells were pulse labeled with [methyl-\(^{3}H\)]thymidine (20 \(\mu\)Ci/ml) for 3 h. At the end of the labeling period the cells were washed with cold phosphate-buffered saline and lysed on the plate with NP-40-containing buffer. After sedimentation of the nuclei for 3 min at 1,000 \(\times\) g, the cytoplasmic fraction was precipitated with trichloroacetic acid and filtered through glass fiber filters, and the insoluble radioactivity was counted in a toluene-based scintillator. Open bars, infected control cells; striped bars, infected enucleated cells. The background incorporation in the cytoplasm of control or enucleated uninfected cells (0.005 and 0.009 cpm/cell; respectively) has been subtracted from all experimental values.

Table 1. Hybridization of pulse-labeled cytoplasmic DNA from ASF virus-infected cells to viral DNA

<table>
<thead>
<tr>
<th>Time of (^{3}H)thymidine labeling (h.p.i.)</th>
<th>Input radioactivity (cpm)</th>
<th>Hybridized radioactivity (cpm)</th>
<th>% Hybridization to ASF virus DNA (5 (\mu)g/filter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-11</td>
<td>857</td>
<td>182</td>
<td>19.3</td>
</tr>
<tr>
<td>11-14</td>
<td>2,228</td>
<td>947</td>
<td>40.7</td>
</tr>
<tr>
<td>14-17</td>
<td>2,838</td>
<td>1,462</td>
<td>49.7</td>
</tr>
<tr>
<td>17-20</td>
<td>3,182</td>
<td>1,044</td>
<td>31.0</td>
</tr>
<tr>
<td>20-23</td>
<td>3,017</td>
<td>694</td>
<td>21.2</td>
</tr>
<tr>
<td>Purified ASF virus DNA</td>
<td>1,603</td>
<td>741</td>
<td>44.4</td>
</tr>
</tbody>
</table>

* A background value of 1.8% corresponding to blank filters has been subtracted from all experimental values.

**DISCUSSION**

The methodology to produce mass cultures of enucleated cells has allowed the study of the implications of the nuclear functions in the development of viruses which replicate and mature in the cytoplasm of the infected cells. This study is important in establishing the virus-host cell interactions that lead to the productive infection.

In this report we present the results of such a study regarding the infection of Vero cells with ASF virus. In none of the experiments performed could a productive infection on enucleated cells be detected under conditions that allowed the development of Sindbis virus.

Recently the importance of the competence of the enucleated cells to support virus growth (9) and the influence of the length of the virus growth cycle in the efficiency of replication in enucleated cells. Considering these possible problems, experiments were performed at 32°C as well as at 37°C with identical results (data not shown).

In addition, experiments such as the one presented in Fig. 3 support the same conclusions; i.e., lack of replication of ASF virus in enucleated cells is not due to cellular impairment, since enucleation up to 10 h p.i. blocked virus development in the same way as enucleation...
prior to infection. Unfortunately, this type of experiment could not be extended to later times after infection due to the fact that cell losses during the enucleation procedure later than 10 h p.i. were too high to enable a meaningful interpretation of the results.

Furthermore, the kinetics of viral DNA synthesis in enucleated cells have been studied in comparison with control cells. As shown in Fig. 2 and Table 1, no viral DNA synthesis could be detected in enucleated cells (either at 37 or 32°C). Taking into account that viral DNA synthesis starts at about 10 h p.i. and extends to about 24 h p.i., it should be expected that enucleated cells, able to support the growth of Sindbis virus, would support ASF virus DNA synthesis as well, if this function were independent of the cell nucleus.

On the other hand, the results shown in Fig. 3 rule out the possibility that the enucleation would affect only an early step in the virus growth cycle, such as adsorption or perhaps uncoating, since enucleation up to 10 h p.i. did not release the inhibition due to enucleation.

Therefore, with the present evidence we propose that ASF virus multiplication requires a nuclear function(s) to allow viral DNA synthesis to occur.

Morphological alterations have been described in the nuclei of infected cells with ASF virus, including chromatin condensation (2, 17) and separation of the two layers of the nuclear membrane bilayer in those regions without attached viral particles (L. Enjuanes, personal communication). The significance of such observations in view of the requirement of a nuclear function(s) for ASF virus replication is not yet clear. Experiments are in progress to identify the nature of such a nuclear function(s).

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LITERATURE CITED


