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Vaccinia Virus Replication

I. Requirement for the Host-Cell Nucleus

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Using cytochalasin B-induced enucleation techniques, we examined the ability of vaccinia virus to replicate in the absence of the host-cell nucleus in several mammalian cell lines. It was found that virus-infected enucleated cells (cytoplasts) prepared from BSC-40, CVC, and L cells were incapable of producing infectious progeny virus. The nature of this apparent nuclear involvement was studied in detail in BSC-40 cells. Modulations designed to maximize cytoplast integrity and longevity, such as reduction of the growth temperature and initial multiplicity of infection, did not improve virus growth in cytoplasts. Sodium dodecyl sulfate-polyacrylamide gel analysis of the [^{35}S]methionine pulse-labeled proteins synthesized in vaccinia virus-infected cytoplasts demonstrated that both early and late viral gene products were being expressed at high levels and with the proper temporal sequence. Vaccinia virus cytoplasmic DNA synthesis, as measured by [^{3}H]thymidine incorporation, peaked at 3 h postinfection and was 70 to 90% of control levels in cytoplasts. However, in the cytoplasts this DNA was not converted to a DNase-resistant form late in infection, which was consistent with the failure to isolate physical particles from infected cytoplasts. Treatment of vaccinia virus-infected cells with 100 μg of rifampin/ml from 0 to 8 h to increase the pools of viral precursors, followed by subsequent removal of the drug, resulted in a threefold increase virus yield. This treatment had no effect on virus-infected cytoplasts. Finally, vaccinia virus morphogenesis was studied under an electron microscope in thin sections of virus-infected cells and cytoplasts which had been prepared at various times during a single-step growth cycle. It was apparent that, although early virus morphogenetic forms appeared, there was no subsequent DNA condensation or particle maturation in the cytoplasts. These results suggest that vaccinia virus requires some factor or function from the host-cell nucleus in order to mature properly and produce infectious progeny virus.

Vaccinia virus, a large complex DNA-containing poxvirus, has been thought to replicate solely in the cytoplasm of susceptible host cells (23). This idea was based on several lines of evidence: vaccinia virus contains or codes for many of the enzymes necessary for DNA synthesis, transcription, and modification (18); virus replicative factories are located exclusively in the cytoplasm (11); and DNA synthesis, as measured by autoradiography, occurs in vaccinia virus-infected enucleated L cells (24). However, other experiments have shown that pretreatment of host cells with either mitomycin C or actinomycin D to inhibit host nuclear functions causes a marked diminution in subsequent vaccinia virus growth (15, 26). Also, there have been indications that some virus-specific DNA synthesis may occur within the nucleus of poxvirus-infected cells (9, 13, 29). Pennington and Follett (22) studied the replication of vaccinia virus in enucleated BSC-1 cells and found that the production of infectious progeny virus was completely blocked. This was accompanied by a considerable reduction in the levels of virus-specific DNA synthesis, protein synthesis, and mature particle formation, and also in the size and number of virus factories. Similar observations have been made concerning the inability of an iridovirus, African swine fever virus, to replicate in Vero cytoplasts (20). It should be pointed out that replication of cytoplasmic RNA viruses such as vesicular stomatitus virus and Semliki Forest virus is essentially normal in enucleated cells (8).

With the recent evidence demonstrating that another group of cytoplasmic DNA viruses, the icosahedral viruses such as frog virus 3, require the nucleus for viral DNA replication and transcription (10), it was of interest to reexamine the so-called cytoplasmic mode of poxvirus replication and the relationship of the apparent nuclear

involvement. In the experiments reported here, we have carefully examined the ability of vaccinia virus to replicate in the absence of the host-cell nucleus. Our results confirm and extend the previous observation that vaccinia virus will not grow in enucleated cells. Furthermore, experimental conditions have been defined under which the levels of early and late viral gene expression in enucleated cells were nearly equivalent to those in control cells, yet no infectious virus was produced. This apparently resulted from aberrant packaging and maturation of virus particles late in the infectious cycle, indicating that some host-cell nuclear function was required to obtain proper vaccinia virus morphogenesis.

MATERIALS AND METHODS

Radioisotopes and chemicals. [^{35}S]methionine (586.4 Ci/mmol) and [*methyl*-^{3}H]thymidine (55.6 Ci/mmol) were purchased from New England Nuclear Corp. Cytosine arabinoside and rifampin were from Sigma Chemical Co. Cytochalasin B was obtained from Aldrich Chemical Co. All gel electrophoresis reagents came from Bio-Rad Laboratories.

Cells and virus. BSC-40 cells, obtained from M. Ensinger, who selected for a derivative of BSC-1 cells which grew well at 40°C, were maintained in Eagle minimal essential medium (MEM) plus 10% heat-inactivated fetal calf serum (ΔFCS). L cells (I. Tamm) were grown in MEM plus 5% ΔFCS. CVC cells (P. Tegtmeyer) were maintained in basal Earle medium (BME) plus 10% ΔFCS.

Vaccinia virus, WR strain, was obtained from the American Type Culture Collection and twice plaque purified on L cells. Purified vesicular stomatitis virus, Indiana strain, was donated by L. A. Ball.

Growth and purification of vaccinia virus. Crude stocks of vaccinia virus were maintained by low-multiplicity passage on L cells. Crude virus stock was trysinized with an equal volume of 0.25% trypsin (GIBCO) at 37°C for 15 min with frequent shaking in a Vortex mixer and then was diluted with ice-cold adsorption medium (1 part Puck's saline, 1 part MEM plus 5% ΔFCS). Confluent monolayers of L cells (10^7 to 3×10^7 cells/100- by 20-mm dish) were infected at a multiplicity of infection (MOI) of 0.01 to 0.1 at 25°C for 3 h. The virus inoculum was then removed, MEM plus 5% ΔFCS was added, and the dishes were incubated at 37°C for 2 to 4 days (or until the microscopic cytopathic effect [CPE] was complete). Infected cells were harvested by centrifugation and then were resuspended in 1 ml of MEM (minus serum)/dish and stored at −70°C. The titer of crude stocks prepared in this way was 10^9 to 3×10^9 PFU/ml.

Purified vaccinia virus stocks were prepared by infecting log-phase spinner L cells (about 5×10^5 cells/ml) at an initial MOI of 1 with crude virus stock and then incubating the infected culture at 37°C for 48 h. Although one might not expect all cells to be initially infected at this MOI, it in fact consistently gave the highest viral yields. Virus was purified from the infected cells by two cycles of sucrose gradient centrifugation as previously described (1) except that virus dispersion by sonic treatment was totally eliminated and replaced with gentle homogenization in a Duall homogenizer. Assuming 1 unit of optical density at 260 nm equals 1.2×10^{10} particles, purified viral pellets were resuspended in 1 mM Tris-hydrochloride (pH 9) to a concentration of 1 mg/ml (2×10^{11} particles/ml) and stored in small portions at −70°C. Virus yields from this procedure were 150 to 300 PFU/cell, and the resultant purified virus had a PFU-to-particle ratio of 1:8 to 1:20.

Plaque assay. Infectious vaccinia virus was assayed in duplicate on confluent 60- by 15-mm dishes of BSC-40 cells. Ten-fold serial dilutions of purified virus were made in phosphate-buffered saline containing 1 mM Mg^{2+}. Crude virus stocks were trypsinized with an equal volume of 0.25% trypsin at 37°C for 15 min and then diluted with adsorption medium. In either case, washed-cell monolayers were infected with 0.5 ml of virus inoculum for 3 h at 25°C with occasional rocking to prevent drying and to obtain a uniform distribution of virus. Virus inocula were removed after 3 h and replaced with MEM plus 5% ΔFCS and 1% agar (Noble). The assays were incubated at 37°C under 5% CO_2 for 2 to 3 days and then were stained with 0.01% neutral red for 2 to 4 h to visualize and enumerate plaques.

Enucleation of cell monolayers. Cell monolayers were enucleated on 60- by 15-mm tissue culture dishes by use of the drug cytochalasin B (3). Slight modifications of previously published procedures (25) were necessary to achieve high-yield mass enucleation of the various cell lines (specific procedures for each cell line are given below). Subconfluent monolayers of cells were inverted aseptically in bottles of 37°C cytochalasin B-containing medium plus serum and preincubated at 37°C. The bottles were then placed in a prewarmed GSA rotor and centrifuged in a Sorvall RC2-B centrifuge at 37°C. After centrifugation, the cytochalasin B-containing medium was replaced with medium plus serum, and the monolayers were put at 37°C until they returned to their normal morphology. Control cells were treated similarly except that they were not centrifuged. The efficacy of enucleation was estimated by Giemsa staining the monolayers and counting the percentage of cells which still retained a nucleus. The cell loss was estimated by both microscopic inspection and counting cells, before and after enucleation, by use of a hemocytometer. Specific details of the procedure used for each cell line are as follows: for L cells, MEM + 5% ΔFCS + 10 μg of cytochalasin B/ml, 15-min preincubation, centrifugation at 8,500 rpm for 45 min, 1-h recovery; for BSC-40 cells, MEM + 10% ΔFCS + 10 μg of cytochalasin B/ml, no preincubation, centrifugation at 8,500 rpm for 20 min, 1-h recovery; for CVC cells, BME + 10% ΔFCS + 50 μg of cytochalasin B/ml, 1-h preincubation, centrifugation at 10,500 rpm for 45 min, 30-min recovery.

Single-step growth experiments. After the recovery interval of the enucleation procedure, monolayers were washed twice with 37°C medium (minus serum) and then infected with 0.5 ml of an appropriate dilution of purified vaccinia virus in phosphate-buffered saline containing 1 mM Mg^{2+}. After 30 min at 25°C, the virus inoculum was removed, and the

monolayers were washed twice with 37°C medium (minus serum). Medium plus serum (4 ml) was added, and the dishes were put at 37°C and 5% CO_2. At the indicated times duplicate dishes of virus-infected cells and cytoplasts were put at −70°C to stop further virus development. The following day the dishes of cells were thawed, and all debris was loosened with the aid of a rubber policeman and an additional 1 ml of medium. The entire infected-cell lysate was collected and refrozen. Infectious virus titers were then determined as described earlier. Experiments measuring virus macromolecular synthesis were carried out similarly except that the monolayers were washed twice with medium (lacking serum and the appropriate metabolite) prior to being labeled with the radioactive precursor.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on 12.5% slab gels by the methods of Studier (28). Small samples of radioactively labeled infected cells were acetone precipitated, dried, resuspended in sample buffer, boiled for 5 min, and then analyzed on 1-mm-thick slab gels. Autoradiography of dried gels was carried out with Kodak RP Royal X-Omat medical X-ray film, and the films were later developed in a Kodak Rapid-Process automatic developing unit.

Electron microscopy. Multiple dishes of BSC-40 cells and cytoplasts (10^6 cells/dish, 98.6% enucleation, 2% cell loss) were infected with vaccinia virus at a MOI of 10. At 0, 3, 6, 9, and 12 h postinfection, cells were removed from the dishes with trypsin-EDTA and harvested by low-speed centrifugation. The cells were suspended in 1 ml of 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4) containing 2 mM $CaCl_2$. After 2 min at 25°C, the suspension was centrifuged, the supernatant fluid was decanted, and the undisturbed pellets were fixed in the above glutaraldehyde for 30 min at 25°C. The pellets were postfixed in 1% osmium tetroxide in 0.15 M cacodylate (pH 7.4) for 30 min and then stained en bloc with 0.5% uranyl acetate in acetate buffer (pH 5.0) for 30 min, dehydrated in acetone, and embedded in Epon 812 (14). Thin sections were cut on a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate (27), and examined in a JEOL 100B electron microscope at 60 kV.

RESULTS

Lack of vaccinia virus replication in enucleated mammalian cells. Cytochalasin B-induced mass enucleation of monolayers of animal cells is a widely used technique which has proven useful in the study of various virus-host cell interactions (25). We have utilized this methodology to enucleate several different cell lines to test the effects of this procedure on the ability of the cells to support a subsequent infection by vaccinia virus. Figure 1 shows the results obtained by infecting CVC, L, and BSC-40 cells and cytoplasts with vaccinia virus at low multiplicity and monitoring infectious virus growth for 24 h postinfection. Low-multiplicity infections, 5 to 10 PFU/cell, were chosen for two reasons. First, it was found that these conditions gave the highest relative virus yield in 24 h. Second, high-multiplicity infections elicited an acute destructive CPE which cytoplasts might

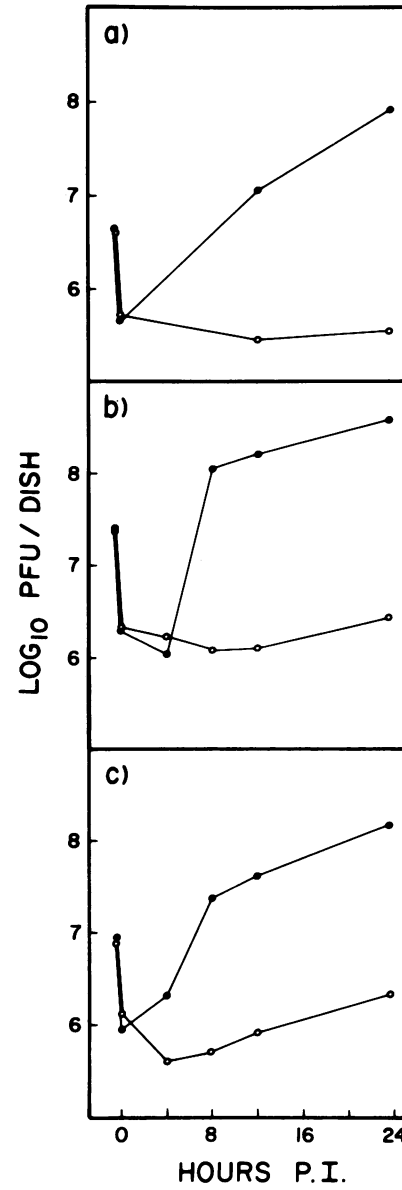

FIG. 1. *Replication of vaccinia virus in control and enucleated mammalian cells. Subconfluent monolayers of intact cells (●) or cytoplasts (○) were infected with vaccinia virus at a MOI of 5. Duplicate dishes were removed at the indicated times, and virus was harvested and titered on BSC-40 monolayers as described. (a) CVC cells (9 × 10^5 cells/dish, 99.8% enucleation, 5% cell loss); (b) L cells (5 × 10^6 cells/dish, 99.5% enucleation, 8% cell loss); (c) BSC-40 cells (1.5 × 10^6 cells/dish, 98.4% enucleation, 3% cell loss).*

have been less able to tolerate and repair than whole cells. It is obvious in Fig. 1 that, although vaccinia virus grew quite well in all three control cells (80 to 105 PFU/cell), it grew poorly if at all in the cognate cytoplasts (0.4 to 1.2 PFU/cell). The small increment of increase in infectious virus titer in the cytoplast preparations could be accounted for by the 1 to 3% remaining nucleated cells, so it is doubtful whether enucleated cells are capable of producing any infectious progeny vaccinia virus. Also, no plaques or foci of infection could be observed on monolayers of cytoplasts infected with 50 to 100 PFU of virus per dish and stained with neutral red at various times postinfection. As a control, the above experiments were repeated with vesicular stomatitis virus which was able to grow to 30 to 100% of control titers, depending on initial MOI, in each of the various cytoplasts, in agreement with previous work (7). One interesting experiment would have been to enucleate vaccinia virus-infected cells at various times postinfection to ascertain when the nucleus was required in the viral growth cycle. Unfortunately, the combination of virus-induced CPE and centrifugation caused the cells to fall off the dish during enucleation at times after 3 h, and thus the above experiment was not technically feasible.

Further studies into the apparent nuclear requirement for vaccinia virus growth were carried out with BSC-40 cells. This system was chosen for a variety of reasons: it afforded ease, rapidity, and reproducibility of the enucleation protocol; the biosynthetic capabilities of BSC-40 cytoplasts were very high, as will become evident later; BSC-40 monolayers demonstrated the highest plaquing efficiency of several cell lines that were tested, so experiments would be carried out and titered on homologous cells; and, finally, results could be compared to earlier work which used a similar cell line, BSC-1 (22).

Experiments designed to rule out possible artifactual results due to side effects from the drug cytochalasin B on vaccinia virus replication were carried out. Pretreatment of BSC-40 monolayers with 10 μg of cytochalasin B/ml or presence of the drug throughout the course of infection only reduced the virus yield by 21 and 32%, respectively. This agrees with earlier studies in which 4 μg of cytochalasin B/ml was used (6). Note that the 1-h pretreatment regimen was the normal control used in most of the experiments reported here. Under a microscope it was apparent that BSC-40 cells rapidly extruded their nucleus in response to cytochalasin B to a point where it was connected to the main body of the cell by only a slender thread of cytoplasm. Even in this gross morphological state, the cells supported vaccinia virus growth, whereas breakage of the cytoplasmic stalk with centrifugal force during the enucleation procedure resulted in a total loss of infectious virus production (Fig. 1).

One trivial explanation for the failure of vaccinia virus to replicate in enucleated BSC-40 cells is that during the period of time required for virus development (9 to 10 h) the cytoplasts deteriorate too rapidly for mature virus to be formed. Several lines of evidence argue against this. First of all, other viruses with generation times similar to that of vaccinia virus grow well in enucleated BSC-1 cells (8), although the complex morphogenetic pathway of vaccinia virus may complicate this sort of analogy. Second, BSC-40 cytoplasts are able to take up and retain the vital dye neutral red for 36 to 48 h. Third, infecting BSC-40 cells and cytoplasts at several MOI (1, 10, or 100) to minimize or maximize vaccinia virus-induced cellular CPE did not raise or lower the ability of cytoplasts to support virus growth, whereas there was an inverse relationship between multiplicity and virus yield in whole cells. Microscopic observations demonstrated that whatever virus function elicited CPE (rounding up and clumping), it was expressed with the same timing and severity in cytoplasts as in whole cells. Finally, carrying out experiments at 31°C, a condition known to prolong the integrity and longevity of cytoplasts (25), did not allow vaccinia virus reproduction in cytoplasts but it did lower the virus yield in whole cells 50% (data not shown).

Expression of both early and late virus functions in enucleated cells. The ability of vaccinia virus to direct its own macromolecular synthesis and to inhibit host-specific events was next examined. Figure 2 shows the results of pulse-labeling vaccinia virus-infected BSC-40 cells and cytoplasts with [^{3}H]thymidine to measure DNA synthesis. Incorporation of the radioactive label showed virtually identical kinetics in both cases, with the maximum rate occurring at 3 h postinfection. At this time, the virus-infected cytoplasts were supporting synthesis of vaccinia virus DNA at about 80% of control levels, although this capability dropped off more rapidly at later times (3.5 to 5 h) in the cytoplast preparations. To ensure that what was being measured was, in fact, vaccinia virus DNA synthesis, an identical experiment was carried out except that cytoplasmic extracts were made at each time point and counted. The results were identical to those in Fig. 2, indicating that virtually all of the radioactive counts were being incorporated in the cytoplasmic fraction, i.e., into viral DNA. The fact that vaccinia virus DNA was synthesized in cytoplasts strongly implies that the virus-coded early functions for DNA biosynthetic enzymes and core uncoating

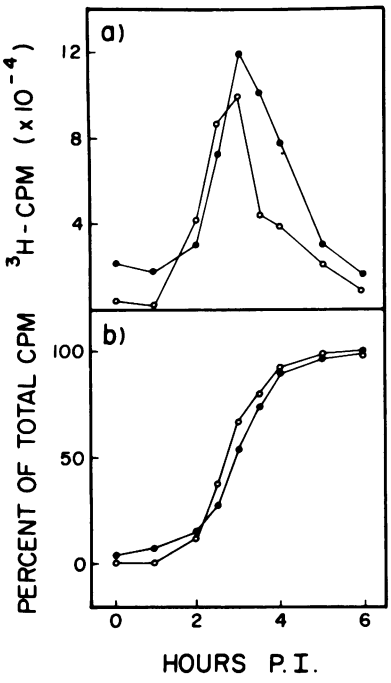

FIG. 2. *Vaccinia virus DNA synthesis in infected BSC-40 cells. Intact BSC-40 cells (●) or cytoplasts (○) (2.75 × 10^6 cells/dish, 98.7% enucleation, 2% cell loss) were infected with vaccinia virus at a MOI of 10. At the indicated times, dishes were pulsed with [^{3}H]thymidine (2 μCi/ml) for 10 min, and then the trichloroacetic acid-precipitable radioactivity per dish was determined. (a) Pulse-labeled vaccinia virus DNA synthesis. (b) Cumulative plot of vaccinia virus DNA synthesis. Note: at 3 h, dishes of uninfected BSC-40 cells and cytoplasts incorporated 154,000 and 1,200 cpm, respectively.*

had been expressed (24). The question of the newly synthesized DNA being transcriptionally active in enucleates was next examined. RNA synthesis was not studied directly, but rather the virus polypeptides being coded for were resolved by SDS-PAGE. In the absence of any stringent translational control, the viral proteins being translated should reflect the amount and types of vaccinia virus mRNA species being transcribed at various times postinfection.

Figure 3 shows the results of [^{35}S]methionine pulse-labeling of BSC-40 cells and cytoplasts infected at high multiplicity (50 PFU/cell) with vaccinia virus. The top panel exhibits some of the kinetic considerations. The first point to be made is that BSC-40 cytoplasts, whether infected or not, initially synthesized protein at 60 to 70% of the rate of control cells and then lost their protein synthetic capabilities slowly throughout the duration of the experiment. In both cells and cytoplasts, a rapid inhibition of host-cell protein synthesis occurred during the first 1.5 h, but this was rapidly obscured as virus-specific proteins began to be made (3 to 8 h). This effect was even more dramatic when the

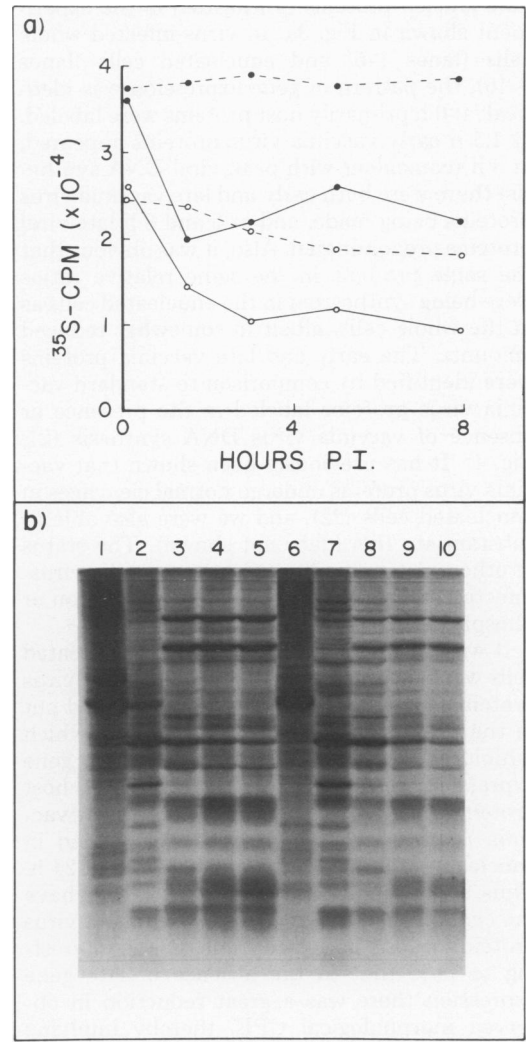

FIG. 3. *Vaccinia virus protein synthesis in BSC-40 cells. Intact BSC-40 cells (●) or cytoplasts (○) (2.4 × 10^6 cells/dish, 98.0% enucleation, 2% cell loss) were infected with vaccinia virus at a MOI of 50. At the indicated times, dishes were pulsed for 20 min with 5 μCi of [^{35}S]methionine/ml. Cells were harvested into 0.5 ml of ice-cold 1 mM Tris-hydrochloride (pH 9). Uninfected cells and cytoplasts were also monitored (dashed lines). (a) Samples of 100 μl were processed to determine hot trichloroacetic acid-precipitable radioactivity. (b) Amounts of 50 μl of the infected samples were analyzed by SDS-PAGE and subsequent autoradiography (lanes 1–5, vaccinia virus-infected BSC-40 cells at 0, 1.5, 3, 5, and 8 h postinfection; lanes 6–10, vaccinia virus-infected cytoplasts at 0, 1.5, 3, 5, and 8 h postinfection).*

infections were carried out at a low multiplicity (5 PFU/cell), in which case virus protein synthesis was only a small percentage of the total.

Figure 3b is an autoradiograph of an SDS-polyacrylamide gel analysis of the [^{35}S]methionine-labeled proteins synthesized in the experiment shown in Fig. 3a. In virus-infected whole cells (lanes 1–5) and enucleated cells (lanes 6–10), the pattern of gene expression was identical: at 0 h primarily host proteins were labeled, at 1.5 h early vaccinia virus proteins appeared, at 3 h (coincident with peak viral DNA synthesis) there were both early and late vaccinia virus proteins being made, and at 5 and 8 h late viral proteins predominated. Also, it was obvious that the same proteins in the same relative ratios were being synthesized in the enucleated cells as in the whole cells, albeit in somewhat reduced amounts. The early and late vaccinia proteins were identified by comparison to standard vaccinia virus proteins labeled in the presence or absence of vaccinia virus DNA synthesis (21; Fig. 4). It has previously been shown that vaccinia virus proteins undergo normal cleavages in enucleated cells (22), and we were also able to substantiate this (data not shown). The status of other protein modifications in vaccinia virus-infected enucleated cells, such as acetylation or phosphorylation, is not yet known.

It was of interest to see how long enucleated cells were capable of synthesizing vaccinia virus proteins. Therefore, infections were carried out in the presence of cytosine arabinoside, which inhibits DNA synthesis and hence late gene expression. Figure 4 shows that, although host protein synthesis was rapidly shut off, early vaccinia proteins continued to be synthesized in enucleated and whole cells for more than 24 h. Thus, it was obvious that enucleated cells have the capability of making large amounts of virus protein for long periods of time. It was interesting to note that in the absence of late gene expression there was a great reduction in observed morphological CPE, thereby implying that this effect was the result of a late gene function.

DNA packaging and viral morphogenesis. The assembly of vaccinia virus constituents into mature infectious particles is a complicated multistep process which has been only partially characterized (17). It was possible that, although vaccinia virus-infected enucleated cells produced all of the necessary viral components, critical catalytic concentrations of one or more may not have been reached, thereby inhibiting morphogenesis. To address this question, we used the drug rifampin, which has been shown to completely block vaccinia virus assembly while allowing continued expression of both

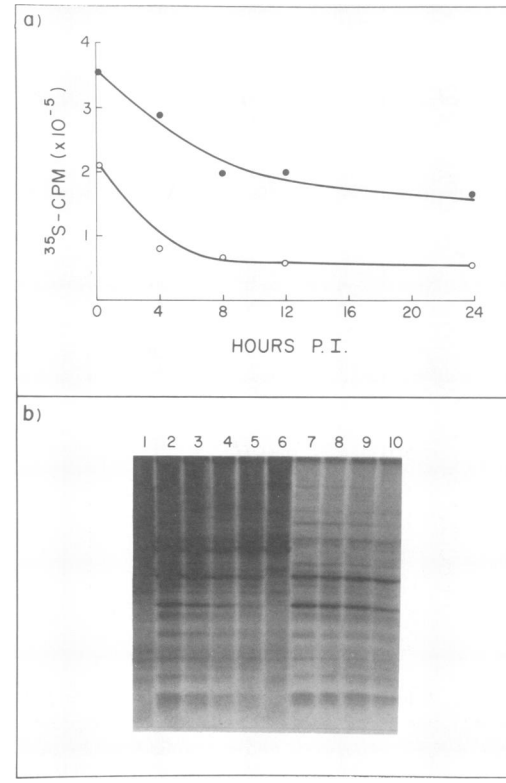

FIG. 4. *Vaccinia virus protein synthesis in the presence of cytosine arabinoside (CAR). BSC-40 cells* (●) *or cytoplasts* (○) *(2.3 × 10^6 cells/dish, 97.6% enucleation, 3% cell loss) were infected with vaccinia virus at a MOI of 50. CAR was present at 25 μg/ml throughout the infection. Dishes were pulsed with 2 μCi of [^{35}S]methionine/ml at the indicated times and analyzed as described in the legend of Fig. 3. (a) Vaccinia virus protein synthesis. (b) SDS-PAGE analysis of [^{35}S]methionine-labeled vaccinia virus proteins (lanes 1–5, 50 μl of virus-infected BSC-40 cells at 0, 4, 8, 12, and 24 h postinfection; 6–10, 100 μl of virus-infected cytoplasts at 0, 4, 8, 12, and 24 h postinfection.*

early and late virus functions (19). Upon drug removal this effect is rapidly reversed, and infectious particles are quickly formed. Table 1 shows the results of such a treatment on vaccinia virus-infected BSC-40 cells and cytoplasts. The rifampin block-reversal step elicited a 3.5-fold increase of virus growth in whole cells. No similar amplification was observed in the cytoplast preparations.

Earlier it was demonstrated that vaccinia virus-specific DNA synthesis occurred at a high rate in infected cytoplasts (Fig. 2), but the subsequent fate of this DNA was not examined. Table 2 shows the results obtained when the viral DNA synthesized from 2 to 4 h postinfection was labeled with [^{3}H]thymidine and then

TABLE 1. *Effect on rifampin on the yield of vaccinia virus from infected BSC-40 cells*[a]

Time of rifampin removal (h)	Control		Enucleated cells	
	PFU/dish	PFU/cell	PFU/dish	PFU/cell
0	3.4×10^7	50.3	3.5×10^5	0.5
8	1.2×10^8	177.8	3.7×10^5	0.6
24	3.6×10^5	0.5	3.3×10^5	0.5

[a] BSC-40 cells and cytoplasts (7×10^5 cells/dish, 99.5% enucleation, 1% cell loss) were infected with vaccinia virus at a MOI of 5 in the presence of 100 μg of rifampin/ml. Drug-containing medium was removed at the indicated times postinfection and replaced with medium alone. At 24 h, infections were stopped and titers were determined as usual. All assays were done in duplicate.

evaluated at various times for packaging, as assayed by conversion of the labeled DNA from a DNase-sensitive to a DNase-resistant form (19). Whereas more than 50% of the viral DNA made in whole cells became resistant to the action of DNase, there was no change in the DNase sensitivity of cytoplast viral DNA in 20 h. This observation was supported by the failure of us and others (22) to isolate physical particles from vaccinia virus-infected enucleated cells. Vaccinia virus infections of BSC-40 cells and cytoplasts were carried out in the presence of [^{3}H]thymidine. The labeled lysates, plus unlabeled carrier virus, were then subjected to the virus purification scheme described in Materials and Methods. Less than 5% of the radioactivity banding with mature virus from control cells was found at a corresponding position from the cytoplast preparation (data not shown). Taken together, these data imply that viral DNA synthesized in the absence of a host-cell nucleus was either never packaged or was packaged in an osmotically fragile form that was disrupted during the DNase assays and particle purification procedures. The exact nature of morphogenetic events happening in vaccinia virus-infected cells and cytoplasts was then examined in an electron microscope.

Figure 5 displays typical examples of virus developmental forms observed in vaccinia virus-infected BSC-40 cells during a single growth cycle experiment. From uncoating to production of new infectious progeny virus 9 to 12 h later, an orderly set of morphogenetic steps can be seen, similar to those previously described (17). Vaccinia virus was taken up and uncoated (Fig. 5A), the cores subsequently broke down and became "viroplasm" or "virus factories" (Fig. 5B), membranes with prominent spicules enveloped "viroplasm" (Fig. 5C), and DNA condensation began at one end (Fig. 5D) and proceeded to the formation of mature particles (Fig. 5E).

Figure 6 demonstrates that this was not true in virus-infected enucleated cells. Although the early steps of morphogenesis up to formation of immature particles (Fig. 6A and B) appeared normal, beyond this development went awry. DNA condensation, if seen at all, was aberrant, giving a mottled appearance to the immature particles (Fig. 6C). At later times (6 to 12 h postinfection), membrane aberrations and bizarre viral forms were apparent (Fig. 6D). Virus development seemed to be arrested at a point analogous to the class J mutants of Dales et al. (5). Also, virus factories of similar size and density were observed in both whole and enucleated cells.

DISCUSSION

Pennington and Follett originally observed that vaccinia virus would not grow in enucleated BSC-1 cells (22). These experiments were carried out at a high MOI under conditions where viral yields in control cells were relatively low and overall viral gene expression and DNA replication in enucleated cells were depressed. It could be argued that this result was due to some factors other than enucleation per se. For example, the high MOI could cause severe CPE which would result in an inhibition of the production of critical concentrations of viral components required for proper maturation in BSC-1 cells. However, we have been able to show that in several cell lines low-multiplicity vaccinia virus infections resulting in good viral growth in cells did not elicit any viral growth in the corresponding cytoplasts (Fig. 1). By all the criteria tested, both early and late viral functions were expressed properly and at relatively high levels in enucleated BSC-40 cells (Fig. 2 and 3), but

TABLE 2. *Conversion of vaccinia virus cytoplasmic DNA to a DNase-resistant form*[a]

Time postinfection (h)	Percent DNase-resistant cpm	
	Control	Enucleated
4	7.6	7.5
8	42.4	5.5
20	52.0	8.6

[a] BSC-40 cells and cytoplasts (3.5×10^6 cells/dish, 98% enucleation, 3% cell loss) were infected with vaccinia virus at a MOI of 10. From 2 to 4 h postinfection, the dishes were labeled with 2 μCi of [^{3}H]thymidine. At the indicated times, duplicate plates were removed, cells were harvested, and a cytoplasmic extract was prepared (19). One half of the extract was precipitated with trichloroacetic acid and counted; the other half was made 50 μg/ml in pancreatic DNase and incubated at 37°C for 30 min prior to trichloroacetic acid precipitation.

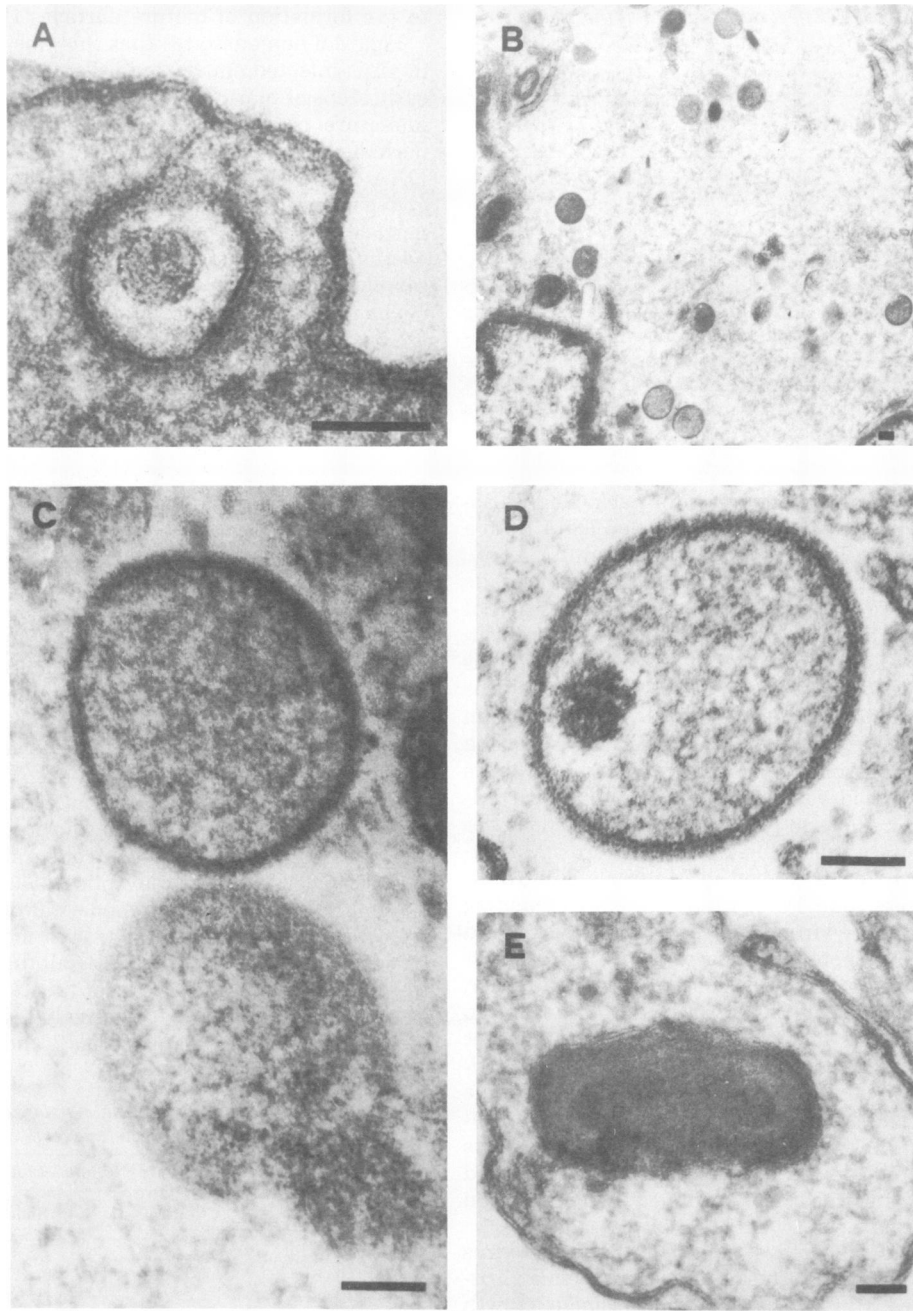

FIG. 5. *Electron micrographs of thin sections of vaccinia virus-infected BSC-40 cells. Displayed are the typical vaccinia virus morphogenetic forms that were observed at the indicated times post infection. (A) Virus particle shortly after penetration, 0 h. (B) Virus factory, 3 h. (C) Immature particles in the process of enveloping DNA, 6 h. (D) Partially condensed DNA within immature particle, 9 h. (E) Mature virus particle, 12 h. Bar = 0.1 μm.*

the viral DNA synthesized in enucleated cells was not converted to a DNase-resistant form as it was in control cells (Table 2). A more detailed electron microscopic study demonstrated that the maturation of the viral components into mature progeny particles was aberrant or absent in enucleated cells (Fig. 5 and 6). In contrast to Pennington and Follett (22), we found no differ-

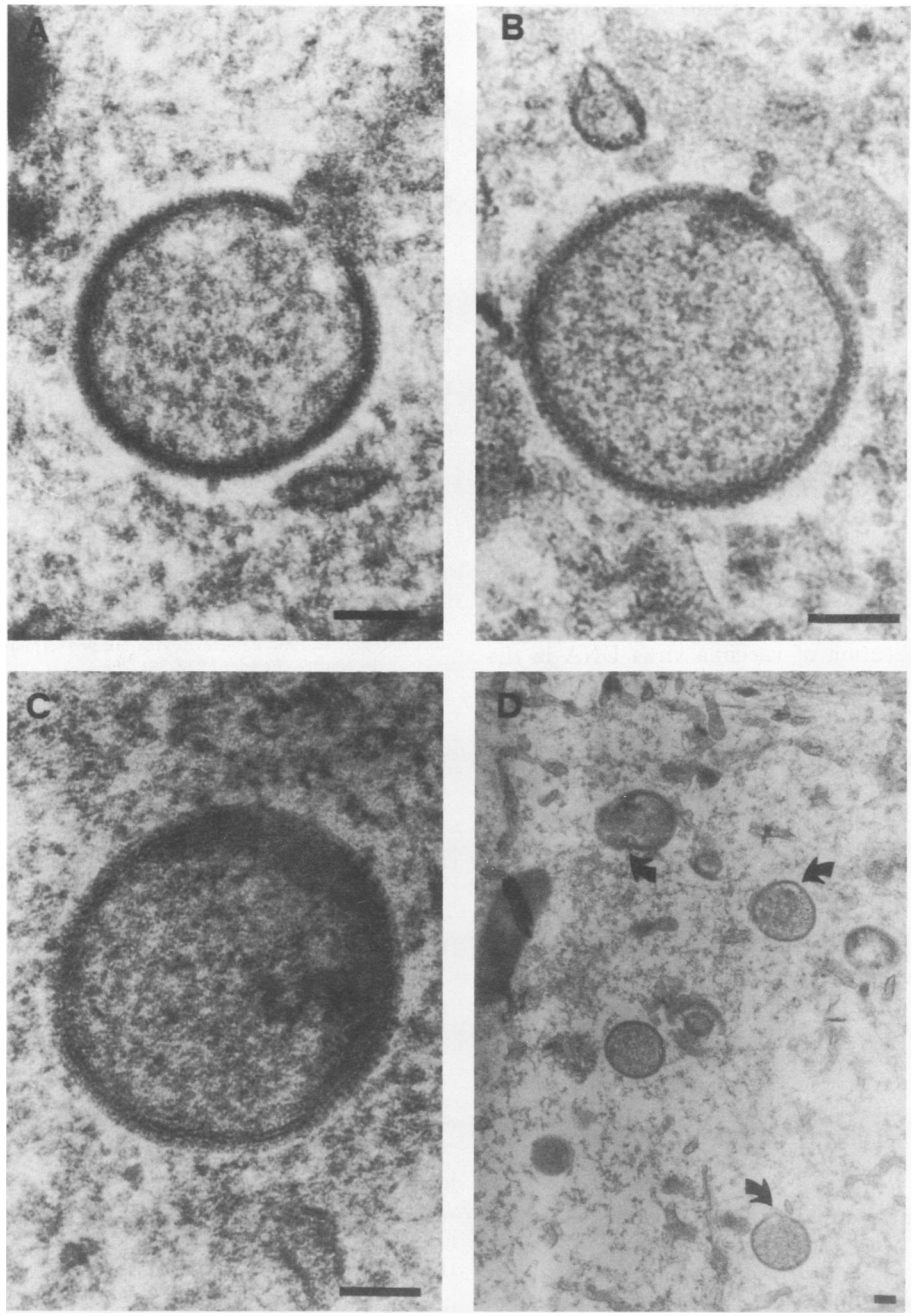

FIG. 6. *Electron micrographs of thin sections of vaccinia virus-infected BSC-40 cytoplasts. The predominant forms observed at the indicated times postinfection are shown. (A) Immature particle enveloping DNA, 3 h. (B) Completely enveloped particle, 6 h. (C) Immature particle exhibiting faulty DNA condensation, 12 h. (D) Immature particles late in infection with aberrant membranes and no apparent DNA condensation, 12 h. Bar = 0.1 μm.*

ence in the number or size of virus factories in the enucleated cells. From the data reported here and from other studies of virus-host cell interactions using enucleated cells (25), it is unlikely that this result is due to the enucleation procedure itself. Therefore, our data support and extend earlier work (22) and indicate that the host nucleus plays some important role in vac-

cinia virus growth. This also agrees with other studies using metabolic inhibitors (15, 26). Thus, it would seen that, like frog virus 3, vaccinia virus can no longer be considered a cytoplasmic virus in the strict sense of the word (23).

If one accepts the notion that vaccinia virus requires the host-cell nucleus in order to replicate, then several interesting questions are raised. First, does vaccinia virus merely require the physical environment of the nuclear body, or must the nucleus be active? Previous studies with phytohemagglutinin-activated leukocytes suggested an active nuclear requirement (16). However, because of the quiescent state of the protein synthetic machinery in resting leukocytes, these results must be viewed with caution. A second question is whether the needed nuclear function is a component or an enzyme and, similarly, whether this function is normally expressed in uninfected cells or is induced by the vaccinia virus infection. One finding which may be pertinent is that of LaColla and Weissbach (13), who showed that some vaccinia virus DNA replication occurs in the nucleus. It is possible that replication of vaccinia virus DNA in the nuclear environment is required for expression of the needed nuclear function. However, the observed absence of UV-enhanced reactivation of subsequent vaccinia virus growth under conditions which stimulate the growth of a bona fide nuclear virus such as herpes simplex virus (2) questions this hypothesis.

Whatever its nature, the requirement for nuclear function becomes apparent during the assembly of vaccinia virus. It may be a situation analogous to the *groE* locus of *Escherichia coli*, where a host-coded function is required for proper phage morphogenesis (4). It is also possible that one or more of the virion components (DNA, RNA, or protein) are synthesized or modified improperly in the absence of the nucleus. Experimentally, this may not be detected until this component is unable to interact productively during assembly, hence faulty morphogenesis.

It is interesting to consider how these data compare with earlier research on the effects of the anti-vaccinia virus drug Virazole or ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (12). This drug allows expression of both early and late viral functions but greatly inhibits production of infectious progeny. It has been speculated that Virazole inhibits some specific late viral function. An alternate explanation is that the drug inhibits the required host nuclear function.

Obviously, much more work remains in the study of vaccinia virus replication and how the virus interacts with both the cytoplasmic and nuclear compartments of the cell. Current research efforts in this laboratory are centered on using intact cells to investigate the molecular nature of the apparent nuclear requirement. This area of research would seem to be important in understanding vaccinia virus-host cell interactions and possibly in elucidating previously undescribed host-cell functions.

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