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Vaccinia Virus Replication in Enucleate BSC-1 Cells: Particle Production and Synthesis of Viral DNA and Proteins

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The growth of vaccinia virus in monolayers of BSC-1 cells enucleated by centrifugation in the presence of cytochalasin B has been studied. No evidence for the production of infectious virus in these cells was obtained, and the production of virus particles was reduced to 8.3% compared wih the yield from cytochalasin-treated, uncentrifuged cells. Virus DNA and early and late polypeptides were synthesized with normal timing in enucleate cells, but in reduced amounts; cleavage of structural polypeptide precursors P4a and Px also occurred in enucleate cells. Factories containing immature virus particles were demonstrated in enucleate cells by electron microscopy; these factories were reduced in number and size compared with those found in cytochalasin-treated, uncentrifuged cells.

Two types of experiments have indicated that the early stages of the vaccinia virus growth cycle proceed in the absence of a functional nucleus. In one experimental system (9), cells were pretreated with dactinomycin, which was removed prior to infection. In such cells, although the synthesis of cellular RNA and DNA was severely depressed, thymidine kinase, viral-specific DNA polymerase, a deoxyribonuclease, and viral DNA were synthesized, and uncoating of viral cores occurred. In a more direct approach, Prescott and co-workers (12, 13) used cell cultures which had been treated with cytochalasin B, a mould metabolite. Exposure of cells to this drug causes nuclear extrusion; after prolonged treatment, a small proportion of cells become enucleate (1). They were able to demonstrate by autoradiography that viral DNA synthesis occurred in the enucleate cells and cell fragments produced by the drug.

Recent work with cytochalasin B has led to the development of a technique for the production of cell monolayers comprised almost entirely of enucleate cells (13; E. A. C. Follett, Exp. Cell Res., in press); this paper describes experiments, using this system, to investigate the role of the cell nucleus during the vaccinia virus growth cycle.

MATERIALS AND METHODS

Materials. [^{3}H]thymidine (18.6 Ci/mmol), [^{35}S]methionine (175 Ci/mmol), and [U-^{14}C]protein hydrolysate (57 mCi/mAtom) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Cytochalasin B was purchased from Ralph N. Emmanuel Ltd., Wembley, England.

Virus, cells, and infection. Vaccinia virus (Evans vaccine strain) was grown and titrated in monolayers of BHK-21 cells, and purified by the method of Joklik (4). BSC-1 cells were grown in Eagle medium (Glasgow modified) containing 10% fetal bovine serum and were infected with purified virus at a multiplicity of 50 PFU/cell in Eagle medium containing 2% fetal bovine serum and 0.02 M $MgCl_2$. Absorption was allowed to continue for 30 min at 38 C. The monolayers were then washed and maintained in Eagle medium containing 5% fetal bovine serum.

Enucleation of BSC-1 monolayers. Plastic petri dishes (~3 cm) containing semiconfluent monolayers of BSC-1 cells were inverted in centrifuge tubes containing 5 μg of cytochalasin B per ml in Eagle medium. The tubes were centrifuged at 9,000 rpm for 20 min at 37 C in an MSE 10 $\times$ 100 angle rotor. The monolayers were then incubated in cytochalasin-free medium for 1 h at 37 C. Monolayers (10 or 20) were enucleated simultaneously; one plate from each batch was stained with Giemsa stain after centrifugation and used to estimate cell loss and the efficiency of enucleation. In the experiments reported here, cell losses during centrifugation varied from 7 to 13%, and the proportion of the cells enucleated ranged from 97.8 to more than 99%.

Labeling of proteins with ^{14}C-labeled amino acids. Cell monolayers were washed twice with warm amino acid-free Eagle medium. Proteins were then labeled by the addition of [^{14}C]protein hydrolysate (5 μCi/ml) contained in amino acid-free Eagle medium. After 15 min of incubation at 38 C, the medium was removed and the cells from each plate were scraped into 0.4 ml of 0.001 M Tris-hydrochloride (pH 9.0). Samples were stored at -70 C prior to electrophoresis.

Pulse-chase experiments. Infected cell monolayers were washed twice with warm Eagle medium lacking methionine. Proteins were then labeled by the addition of 50 μCi of [^{35}S]methionine per ml in methionine-free Eagle medium. After 15 min at 38 C, the label was removed and the cells were washed and incubated further in Eagle medium containing 100 times the normal amount of methionine. At appropriate times, cells were scraped into 0.001 M Tris-hydrochloride (pH 9.0) and analyzed by electrophoresis.

Gel electrophoresis and autoradiography. Samples were reduced and dissociated by boiling for 90 s in 1% mercaptoethanol-2% sodium dodecyl sulfate (SDS). After the addition of sucrose and tracking dye, the samples were subjected to electrophoresis in 9% acrylamide gels (10 cm long) by using the discontinuous SDS buffer system described by Laemmli (8). After electrophoresis, the gels were stained with Coomassie brilliant blue, and sliced and dried by the method of Fairbanks et al. (2). Autoradiography was done with Kodak Blue Brand Medical X-ray film.

Electron microscopy. Infected cells were removed from petri dishes by treatment with trypsin-versene for 15 min at 37 C, pelleted by low-speed centrifugation, and fixed for 30 min in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and 15 min in 1% osmium tetroxide. The cell pellets were then dehydrated in alcohol and embedded in Epon by standard procedures. Thin sections were cut on an LKB Ultratome 1 and stained with uranyl acetate and lead hydroxide before examination in a Siemens 101 electron microscope.

RESULTS

Virus growth and particle production. No significant increase in virus titer was observed after the infection of enucleate cell monolayers, whereas virus growth proceeded normally in cells treated with 5 μg of cytochalasin per ml for 40 min at 37 C prior to infection (Fig. 1). Virus plaque production was not observed in enucleate cell monolayers; it was unaffected by cytochalasin treatment of monolayers prior to infection.

Mature virus particle formation in enucleate cells was investigated further by sucrose density gradient analysis of virus purified (with added unlabeled carrier virus) from cytoplasmic homogenates of cells labeled throughout infection with [^{3}H]thymidine. It will be shown later that virus DNA is synthesized in enucleate cells. Typical results are shown in Fig. 2, which demonstrates that virus particle production in enucleate cells is grossly reduced. In another experiment, virus particle production was estimated by measuring the ratio of radioactivity to optical density units of [^{3}H]thymidine-labeled virus purified with a large excess of added carrier virus from enucleate and normal cell homogenates, as described in the legend to Fig. 2. A virus yield of 8.3% from enucleate compared with normal cells was calculated. This is considerably greater than the particle yield expected from the small proportion of nucleated cells remaining in the enucleated monolayer (1.1% in this experiment), and it was concluded that mature virus particles are produced in enucleate cells, albeit in considerably reduced amounts.

Electron microscopy. In nucleate cells treated with cytochalasin, extensive factories were found in almost every cell section examined, and all the commonly observed forms of particle associated with poxvirus maturation were present—complete immature particles, empty immature particles, and arcs of typical immature particle membranes being apparent in all factories (Fig. 3a). Numerous immature particles with dense nucleoids and complete mature virus particles were also evident.

Enucleate cells, in contrast, contained significantly fewer factories, with only the occasional cell section showing evidence of virus growth. Where evident, these factories were much smaller than those in nucleate cells and usually contained less than 10 immature forms per section. Immature particles and membranes were formed apparently normally (Fig. 3b), but only a very small number of morphologically mature virus particles were found.

Virus DNA synthesis. Autoradiographic studies have shown that factories of vaccinia

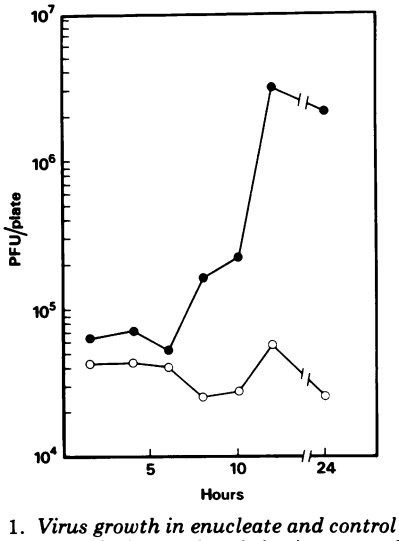

FIG. 1. *Virus growth in enucleate and control cells. At the indicated times after infection, monolayers were scraped from the plates with a rubber policeman. Cells were then disrupted by sonic treatment, and the virus was titrated. Control cells were treated with cytochalasin (5 μg/ml, 20 min at 37 C) but not centrifuged. Each point shows the titer of pooled duplicate samples. Symbols: ●, virus titer, control cells; ○, virus titer, enucleate cells.*

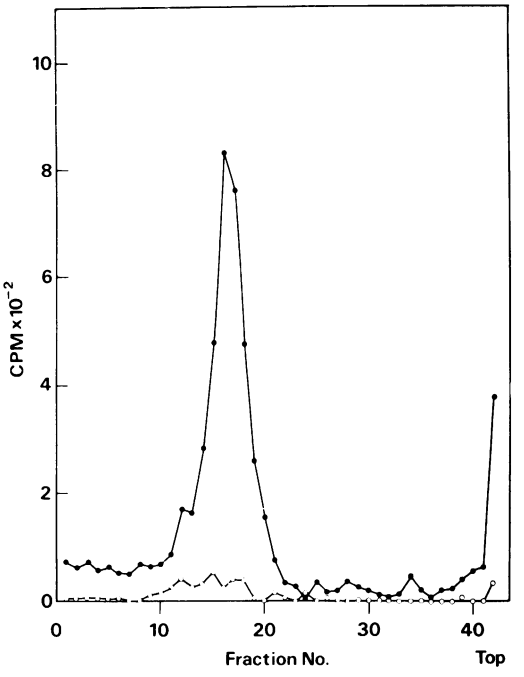

FIG. 2. *Virus particle production in enucleate and control cells. Infected cells were incubated in medium containing 10 μCi of [³H]thymidine per ml throughout infection. At 22 h after infection, the cells were disrupted by sonic treatment and treated with 50 μg of pancreatic deoxyribonuclease per ml for 1 h at 37 C. Carrier, unlabeled vaccinia virus was added, and the virus was purified by the method of Joklik (4), culminating in centrifugation through 20 to 40% sucrose gradients in 0.001 M Tris-hydrochloride, (pH 9.0) for 22 min at 15,000 rpm in a Spinco SW25.1 rotor. The acid-insoluble radioactivity in 0.1-ml samples from each fraction was determined. In both gradients, the radioactivity and optical density peaks were coincident. Symbols: ●, virus from cells treated with cytochalasin but not centrifuged; ○, virus from enucleate cells.*

virus DNA synthesis are set up in enucleate cells or cell fragments (12). We confirmed these observations both by autoradiography and by directly measuring the incorporation of [³H]thymidine into acid-insoluble material at various times during the virus growth cycle (Fig. 4). The time course of virus DNA synthesis was similar to that in normal cells, although a two- to threefold reduction in the amount synthesized was observed. Uninfected enucleate cell DNA synthesis was, as expected, reduced to very low levels (Fig. 4). Total cellular DNA synthesis was measured in this experiment; similar results were obtained in studies which estimated the quantity of radioactively labeled DNA in the cytoplasm of infected cells by the technique of Joklik and Becker (5).

Virus polypeptide synthesis. Figure 5 shows autoradiograms of polyacrylamide gels of ^{14}C-labeled amino acid polypeptides from infected enucleate and cytochalasin-treated but uncentrifuged cells. Comparison of the two autoradiograms shows that the position of bands in gels of identical time samples, the relative intensity of bands in such gels, and the timing of the appearance and disappearance of bands during the virus growth cycle are identical, although the total incorporation of radioactivity is reduced in all the enucleate cell samples, including those from uninfected cells (Fig. 6). This reduction varied from 25 to 44%. It is apparent from the autoradiograms that the decline of host protein synthesis and the sequential development of early and late virus polypeptides proceed normally in enucleate cells.

Comparison of polyacrylamide gel autoradiograms of samples from infected normal and cytochalasin-treated, uncentrifuged cells showed no differences (result not shown); a detailed description of the results obtained with normal cells will be present in a subsequent publication (manuscript in preparation).

Cleavage of virus structural proteins. Several late virus polypeptides are cleaved after synthesis, the major virion core polypeptide precursors P4a and Px being processed in this way at a late stage of virion assembly (6, 7, 10). Pulse-chase experiments showed that these events occurred in enucleate cell (Fig. 7) core polypeptide 4a, which is derived from polypeptide P4a, appearing in increasing amounts during the chase period. The cleavage product of polypeptide Px co-electrophoreses with polypeptide 4b, and an increase in intensity of this composite band is also evident in gels of enucleate cell samples taken during the chase period.

DISCUSSION

In this study, we have shown that the synthesis of vaccinia virus DNA and early and late polypeptides occurs with normal timing in enucleate cells. These results provide strong evidence favoring the viral genome as the source of the code for the virus-induced polypeptides detected in polyacrylamide gel autoradiograms. Both DNA and protein are made in reduced amounts. This reduction cannot be accounted for solely by loss of cells during enucleation, as demonstrated by the small number and size of viral factories in enucleate cells, and may possibly be a consequence of the decreased rate of protein synthesis observed in uninfected cells after enucleation (E. A. C. Follett, Exp. Cell Res., in press). We assumed, but have not investigated, that the uptake and metabolic

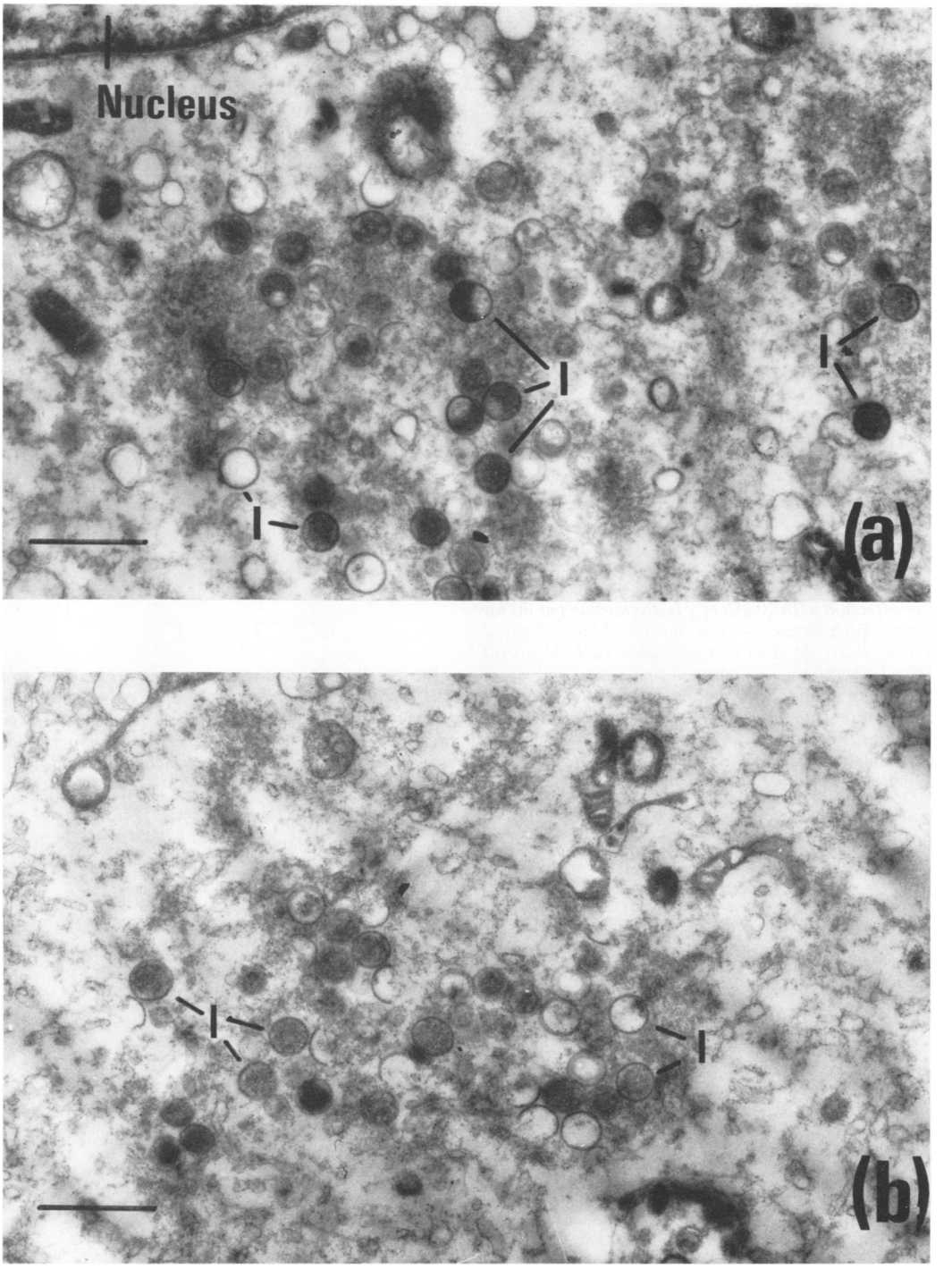

FIG. 3. *(a) Thin section through factory in nucleate BSC-1 cells 17 h after infection with vaccinia virus. Numerous immature particles (I) in varying stages of development are present. Bar ≡ 1 μm. (b) Thin section through factory in enucleate BSC-1 cells 17 h after infection with vaccinia virus. The factory is less extensive than that in Fig. 3a, but typical immature particles and arcs of membrane are present. Bar ≡ 1 μm. Efficiency of enucleation 98.4%.*

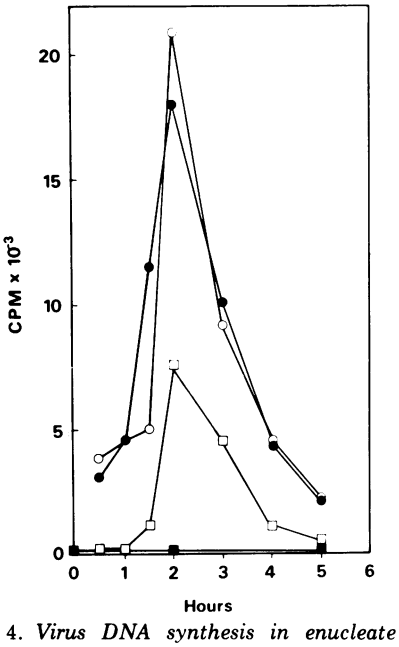

FIG. 4. *Virus DNA synthesis in enucleate and control cells. Cells were labeled at the indicated times after infection with 10 μCi of [³H]thymidine per ml for 15 min. Each monolayer was harvested into 0.5 ml of ice-cold 0.001 M Tris-hydrochloride (pH 9.0), and 0.05-ml samples were applied to filter paper disks which were washed with cold trichloracetic acid prior to determination of radioactivity. Symbols: ○, infected control cells; ●, infected cells pretreated with cytochalasin but not centrifuged; □, infected enucleate cells; ■, uninfected enucleate cells.*

processing of DNA and protein precursors are normal in enucleate cells.

The large reduction in mature virus particle production in enucleate cells can be explained in part by the observed reduction in rate of viral macromolecular synthesis in these cells; further investigations will be required to assess the possibility that cell enucleation may interfere in a more specific way with virus growth. It is possible, for example, that virus DNA synthesis in enucleate cells may be abnormal in some way and that a nucleus is required for the production of mature DNA. We have been unable to find evidence of specific blocks to virus assembly prior to immature virus particle formation or

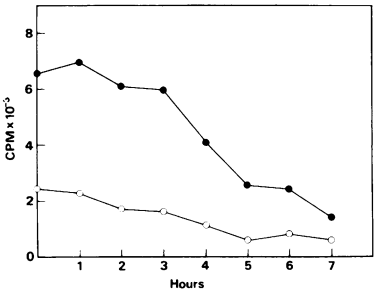

FIG. 6. *Protein synthesis in enucleate and control cells infected with vaccinia virus. Acid-precipitable radioactivity was estimated at various times after infection by using samples from the experiment shown in Fig. 5. Symbols: ●, cells treated with cytochalasin but not centrifuged; ○, enucleate cells.*

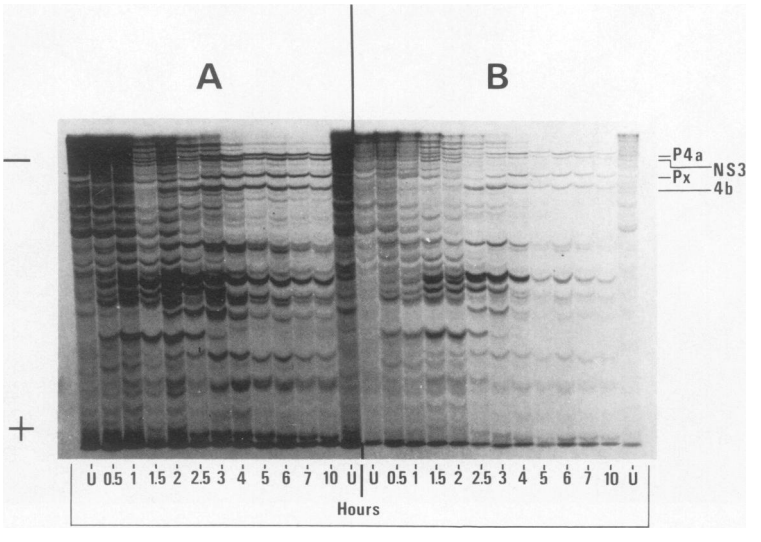

FIG. 5. *Polyacrylamide gel autoradiogram showing virus polypeptide synthesis in enucleate and control cells. At the indicated times after infection, cells were labeled for 15 min with ¹⁴C-labeled amino acids. A, Cells treated with cytochalasin but not centrifuged; B, enucleate cells. U, Uninfected cells. The position of some late virus polypeptides are indicated (P4a, NS3, Px, 4b); see reference 9 for nomenclature.*

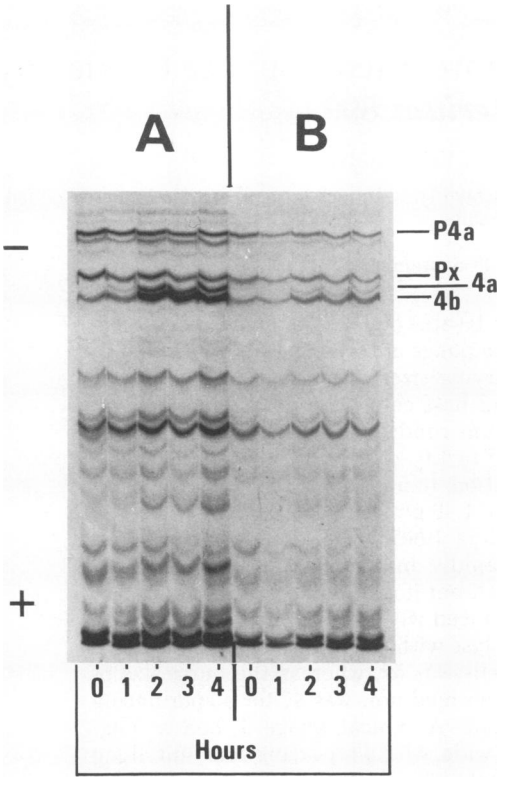

FIG. 7. *Polyacrylamide gel autoradiogram of samples from pulse-chase experiment. Cells were labeled for 15 min at 6 h after infection with [^{35}S]methionine, and chase samples were taken at the times indicated. (A) cells treated with cytochalasin but not centrifuged; (B) enucleate cells.*

the cleavage of virion polypeptide precursors. It is interesting to note, however, that the growth of vesicular stomatitis virus proceeds with only a moderate degree of inhibition in BSC-1 cells enucleated by the method used in this study (3), whereas the yield of infectious poliovirus from cells enucleated by a similar technique is reduced fivefold compared with that from normal cells (11). The growth of echovirus type 1 in enucleated BSC-1 cells is also reduced considerably (C. R. Pringle, personal communication).

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