Virus Replication in Enucleate Cells: Vesicular Stomatitis Virus and Influenza Virus

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The requirement of the presence of a nucleus for the replication of vesicular stomatitis virus and influenza virus has been examined by following the growth and development of these viruses in enucleate BS-C-1 cells. Vesicular stomatitis virus replicates normally in enucleate cells with the rate of production of infectious virus, the amount of virus-specific protein synthesis, and the type of proteins produced being essentially the same in nucleate and enucleate cells. Influenza virus does not replicate in enucleate cells, no virus gene products can be detected, and there is no inhibition of cellular protein synthesis.

The functions of the nuclei of infected cells which are specifically required for the efficient replication of animal RNA viruses have largely been inferred from studies of the effects of various antimetabolites on the synthesis of virus components and the production of infectious virus. On the basis of the results of these investigations, viruses can be divided into three main groups: those which require both nuclear DNA replication and transcription during virus replication, such as the RNA tumour viruses; those which require nuclear DNA transcription, but not DNA replication—the influenza viruses; and those which replicate efficiently even when DNA replication and transcription are completely inhibited, such as picornaviruses, group A arboviruses, and rhabdoviruses.

With the development of procedures for enucleating cells in tissue culture (2-4, 7), it has become possible to directly investigate the requirement for the cell nucleus and its contents in virus replication (6), and in this study the replication in enucleated cells of representative viruses of the latter two groups, an influenza virus and a rhabdovirus, was examined.

MATERIALS AND METHODS

Materials. L-[³S]methionine (35 [³H]Ci/mmol), L-[³H]leucine (58 Ci/mmol), and L-[¹⁴C]leucine (0.31 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. Cytochalasin B, manufactured by Imperial Chemical Industries, Ltd., was obtained from Ralph N. Emmanuel Ltd., Wembley, England.

Viruses. Two influenza viruses, the Rostock strain of fowl plague virus and Ao/Bel/42, were used. The rhabdovirus used was the Cocal strain of vesicular stomatitis virus (VSV). The influenza viruses were

propagated in the allantoic cavity of 10-day-old embryonated eggs, and infected egg allantoic fluid was used as the inoculum in all experiments to give an infectious dose of approximately 100 egg infectious units/cell. VSV was grown in BHK21/C13 fibroblasts, and the culture fluid of these infected cells was used as inoculum.

Cells. Virus replication was examined only in BS-C-1 cells (5). The cells were plated out at a density of 2×10^5 cells/dish (3 cm) and allowed to grow overnight at 37°C in Eagle medium supplemented with 10% fetal bovine serum. Enucleation of the cells in the resulting monolayer was accomplished by inverting the base of the petri dish in a centrifuge tube in medium containing 5 µg of cytochalasin B per ml and centrifuging for 20 min at 9,000 rpm in an MSE Superspeed 65 or 75 ultracentrifuge with the temperature of medium and rotor as near 37°C as possible. A maximum of 10 dishes could be enucleated under identical conditions at any one time. After enucleation, the cells were left to recover for 1 h in normal medium at 37°C before virus infection. One dish from each batch of 10 dishes was stained with Giemsa after the 1-h recovery period, and the efficiency of enucleation (percentage of cells enucleated) was determined by counting more than 500 cells from at least six separate areas of the dish. This technique consistently achieved an efficiency of enucleation of better than 97%. Full details will be published elsewhere (E. A. C. Follett, Exp. Cell. Res., in press). Two types of control were included in experiments, normal cells which were not at any time exposed to cytochalasin B and cells which were exposed to the drug for the same time as the enucleate cells, but which were not centrifuged and hence not enucleated.

Virus assay: VSV-infectivity assay. Monolayers of BS-C-1 cells were inoculated with approximately 1 PFU/cell. After 30 min of adsorption at 37°C, the inoculum was removed by two washes with 5 ml of Eagle medium, and incubation continued at 37°C in

Eagle medium supplemented with 10% fetal bovine serum. Virus released into the medium was assayed at intervals after infection by plaque assay in BHK21/C13 cells at 37 C.

VSV-protein synthesis. On infection with VSV, several virus-specified polypeptides are produced which are readily distinguished by their mobility in polyacrylamide gels (11). The polypeptides produced after VSV infection of enucleate cells and cells treated with cytochalasin B, but not enucleated, therefore were compared.

The medium from enucleated and cytochalasin-treated cells was removed 1 h postnucleation, and the cells were infected with VSV at a multiplicity of 1 PFU/cell. After 30 min of adsorption at 31 C, the virus inoculum was removed by washing, and the original medium was replaced. Incubation was continued for 5 h at 37 C. L-[³H]leucine (6 μ Ci/ml) or L-[¹⁴C]leucine (4 μ Ci/ml) were added at 2 h after infection, and the cells were extracted at 5 h with 2% sodium dodecyl sulfate (SDS) 0.2% dithiothreitol, and 1 M urea in sodium phosphate buffer, pH 7.4. Samples of enucleate and control cultures were mixed and compared by co-electrophoresis in 10% SDS-polyacrylamide gel as described previously (11). For autoradiography, cells were infected similarly, and [³⁵S]methionine (2 μ Ci/dish) in methionine-free medium was added at 3, 6, and 9 h. After 15 min, the proteins were extracted for polyacrylamide gel electrophoresis and autoradiography as described previously (9).

VSV-RNA synthesis. The distribution of RNA synthesis in an enucleated preparation at intervals after infection was examined by labeling the cell monolayer with ³H-uridine (20 μ Ci/dish) immediately after the adsorption period and determining the location of any isotope taken up into acid-precipitable material by autoradiography (E. A. C. Follett, Exp. Cell. Res., in press).

Influenza virus. Neither fowl plague nor the Bel influenza strain produce complete infectious particles in BS-C-1 cells, although all of the virus-specific proteins are made. Consequently, virus development in these cells was studied by following the development of virus-specified proteins by polyacrylamide gel electrophoresis and autoradiography. Infection of cells, radioactive labeling conditions, polyacrylamide gel electrophoresis, and autoradiography were as described previously (9).

RESULTS

VSV: (i) virus growth. VSV replication in BS-C-1 cells treated with cytochalasin B, but not enucleated, was rapid and efficient, the logarithmic phase of virus replication and release occurring between 3 and 8 h after infection. Under these conditions, approximately 1,000 infectious particles were produced per infected cell. In enucleated cells, the production of infectious virus occurred at the same rate and to essentially the same extent as in the cytochalasin-treated nucleate control cells (Fig. 1), although there was a small decrease in virus

yield in enucleated cells compared with the controls. This decrease has been reproducibly obtained in a series of experiments, the average decrease in yield measured in a series of six experiments being ~30%. The only difference noted between the effects of VSV in nucleate and enucleate cells was that a delay in cytopathic effect of about 2 h was consistently observed in enucleate cells. There was no comparable delay in virus release (Fig. 1).

(ii) VSV-RNA synthesis. RNA synthesis in these enucleated preparations is extremely low and can be attributed largely to the few remaining nucleate cells. Autoradiographs have demonstrated significant incorporation in only the remaining nucleate cells (E. A. C. Follett, Exp. Cell Res., in press). After infection with VSV, extensive incorporation of [³H]uridine into enucleate cells was observed as early as 2 h postinfection. At this and later times, all enucleate cells in a preparation showed incorporation, and there was no evidence to indicate that any fraction of the enucleate cells was resistant to infection and thus could be responsible for the decrease in total yield noted above.

(iii) Virus protein synthesis. The synthesis of virus-specific proteins during virus replication in cultures of both normal and enucleate cells was determined by analyzing extracts of infected cells labeled with [³⁵S]methionine and [³H]leucine by polyacrylamide gel electrophoresis either alone or followed by autoradiography. A typical profile is shown in Fig. 2; the cells were infected 2 h postnucleation and labeled from 0 to 4 h. The five viral polypeptides, designated L, G, N, NS, and M (10), were resolved in the treated sample and also in the enucleate sample. This similarity between events occurring in nucleate and enucleate cells was even more clearly demonstrated when gel electrophoresis was followed by autoradiography. A comparison of the polypeptides synthesized at various times postinfection in normal, treated, and enucleate cells (Fig. 3) showed that the time at which synthesis of the five proteins occurred, the amount of each protein made, and the extent of inhibition of host protein synthesis were similar in all types of cells. This was especially the case at 6 h postinfection when no difference could be detected between gels of normal, treated, and enucleate cells.

Influenza virus: (i) infection of BS-C-1 cells. Under the conditions of infection described in Materials and Methods, by using infected-egg allantoic fluid as inoculum, neither of the two influenza viruses used in these studies (fowl plague virus and Ao/Bel/42) were able to replicate in BS-C-1 cells. Instead, both

FIG. 1. The growth of vesicular stomatitis virus (VSV) in enucleate BS-C-1 cells. Enucleate BS-C-1 cells in monolayer culture were infected with VSV (1 PFU/cell), and at the times indicated the virus released into the culture medium was determined by plaque assay in BHK21/C13 cells (O). In the same experiment cells in duplicate cultures were exposed to cytochalasin B for the same time as the cells being enucleated, but they were not centrifuged and thus not enucleated. These cells were likewise infected, and the virus yield was determined (●). Efficiency of enucleation was 98.6%.

FIG. 2. Vesicular stomatitis virus (VSV) protein synthesis in enucleate cells. Proteins labeled between 2 and 5 h in the infected cells were extracted and analyzed in 10% sodium dodecyl sulfate-polyacrylamide gel. The gels were 8 cm long and were run for 6 h at 6 mA/gel. ^3H -labeled proteins synthesized in enucleate cells (●) were compared with ^{14}C -labeled protein (O) from cytochalasin B-treated cells. The major proteins synthesized in enucleate cells correspond to the five VSV proteins, L, G, N, NS, and M. Efficiency of enucleation was 98.2%.

viruses underwent an abortive cycle of replication during which virus-specific proteins were synthesized and soluble hemagglutinin was produced (20 to 40 hemagglutinin units/culture at 8

FIG. 3. The synthesis of polypeptides in vesicular stomatitis virus (VSV)-infected enucleate cells. Enucleate cells were infected with VSV (1 PFU/cell), and at 3, 6, and 9 h after infection, [^{35}S]methionine (2 $\mu\text{Ci}/\text{culture}$) in medium lacking unlabeled methionine was added to separate cultures of normal (N), treated, infected (exposed to cytochalasin, but not centrifuged) (T), and enucleated, infected cells (E) for 15 min. The cells were processed for electrophoresis as described previously (9) and run on 15-cm polyacrylamide gels (11.5%) for 20 h at 2 V/cm. The direction of migration was toward the anode from top to bottom. The virus-specific polypeptide species are designated L, G, N, NS, and M in descending order of molecular weight. Efficiency of enucleation was 98.3%.

h postinfection). The synthesis of influenza virus proteins in infected cells was readily detected by using the same procedures as those described above for VSV. The results shown in Fig. 4 indicate that polypeptides of mobility similar to all of the primary gene products, which are formed during the permissive infection of chicken embryo fibroblasts with fowl plague virus (9), were produced in BS-C-1 cells infected with either strain of influenza. The only notable difference between these products and those detected in the permissive system was that independently of the length of pulse with [^{35}S]methionine, the hemagglutinin precursor, polypeptide 3, was not cleaved and, therefore, the virion hemagglutinin glycopolypeptides were never observed.

(ii) **Infection of enucleated cells.** The results described above demonstrate that in the absence of virus replication the procedures em-

FIG. 4. The synthesis of polypeptides in influenza virus-infected BS-C-1 cells and in infected, enucleate cells. Enucleate cells, normal cells, and cells which had been exposed to cytochalasin but not centrifuged were infected with either Ao/Bel/42 (B) or fowl plague virus (F) (100 egg infectious units/cell). At 3, 6, and 9 h after infection, [35 S]methionine (2 μ Ci/culture) in medium lacking unlabeled methionine was added to each culture for 15 min. The cells were processed for electrophoresis and run under conditions identical to those in Fig. 3. From left to right are shown the polypeptide components from enucleate cells (E), normal cells (N), and cytochalasin-treated cells (T) after mock infection (U), infection with fowl plague (F), and Ao/Bel/42 (B) at the three time points. The virus-specific polypeptides are designated in descending order of molecular weight, P1, P2, 3, NP, MP, 8, and 9 (9). Efficiency of enucleation was 99.3%.

ployed to detect virus-specific protein synthesis were convenient and efficient methods for determining the expression of the genetic information of influenza viruses. In order to examine the effects of influenza virus infection of enucleated BS-C-1 cells, therefore, the proteins synthesized after infection were analyzed. The results shown in Fig. 4 indicate that, although cellular protein synthesis continued after enucleation, the synthesis of virus-specific proteins could not be detected in infected enucleate cells, at least up to 9 h after infection. In addition, the inhibition of cellular protein synthesis, which was evident in infected control cultures and which occurred concomitantly with virus-specific protein synthesis, was not observed in infected enucleated cells. By this criterion, therefore, and unlike VSV, fowl plague virus and Ao/Bel/42 influenza viruses appeared to be inactive in enucleated cells. It should be noted here that incubation of BS-C-1

cells in medium containing cytochalasin B for 1 h before infection did not inhibit subsequent virus protein synthesis (Fig. 4). The results obtained by using enucleated cells, therefore, were not a consequence of an irreversible effect of cytochalasin B treatment on the cells, but were clearly due to the enucleation process. In order to demonstrate this more conclusively and in attempts to gain further information on the role of the nucleus during influenza virus replication, the following experiments were carried out. BS-C-1 cells were infected with fowl plague virus, and at various times after infection they were treated with cytochalasin B and enucleated. After this procedure, they were incubated as normally, and the proteins which they synthesized were analyzed at intervals thereafter. Cells enucleated at either 2 or 4 h after infection continued to produce virus proteins (Fig. 5), albeit at a reduced rate, at least up to 3 h later (i.e., 5 and 7 h postinfection, respectively).

FIG. 5. The synthesis of polypeptides in fowl plague virus-infected BS-C-1 cells enucleated at either 2 or 4 h after infection. Cells were infected with fowl plague virus (1,000 egg infectious units/cell), and at either 2 or 4 h after infection separate cultures were treated with cytochalasin B for 1 h, enucleated (the procedure was organized to be complete by 1 h), or maintained as control cells. The control infected cells were pulsed with [35 S]methionine as described in Fig. 3 at 2, 4, and 6 h after infection (A, B, and C, respectively). The cells treated with cytochalasin B and enucleated at 2 h after infection were pulsed with [35 S]methionine at 5 h after infection (D and E); and the cells treated or enucleated at 4 h after infection were pulsed with [35 S]methionine at 7 h after infection (F and G). Other experimental details were as described in Fig. 3, and the polypeptides are designated as in Fig. 4. Efficiency of enucleation was 98.3%.

Although it is possible to argue from quantitative data obtained from scanning these gels in a densitometer that the small amount of synthesis observed at 5 h (gel E) was caused by only the remaining 1 to 2% nucleate cells, such an argument cannot be applied to the sample enucleated at 4 h postinfection and labeled at 7 h (gel G). At this time polypeptide synthesis was almost 20% of that occurring in the treated sample (gel F), whereas the number of nucleate cells was again 1 to 2% of that in the treated sample. Clearly then, the synthesis of influenza virus proteins can occur in cells treated with cytochalasin B and in enucleated cells provid-

ing that the nuclei are removed at times after infection when the synthesis of virus macromolecules has already begun.

DISCUSSION

The results presented here allow two main conclusions. The replication of VSV as judged by the rate of production of infectious virus, the amount of virus specific protein synthesis, and the type of proteins produced is equally efficient in enucleated and in normal BS-C-1 cells. This virus, therefore, appears not to require any function of the host cell nucleus for replication and clearly does not even require the high degree of cytoplasmic organization present in a normal, nucleate cell.

There is a small decrease in virus yield, and several factors could contribute to this. Some cell loss occurs during enucleation. This has been estimated to be, at most, 13% (E. A. C. Follett, *Exp. Cell Res.*, in press). Treatment with cytochalasin and centrifugation are unlikely to be significant factors, for we have found that neither treatment has any effect whatever on the ability of VSV to replicate in L cells. On leaving the cell, the nucleus detaches a large piece of cytoplasmic membrane. The total area of membrane available for virus maturation is hence considerably reduced. Finally, enucleation must result in extensive disruption of cellular organization. It is thus not surprising that some decrease in virus yield is observed: it is surprising that VSV grows so well in such a system. Several other cytoplasmic RNA viruses have been examined (echovirus, type 1, reovirus, type 3, measles, and respiratory syncytial virus), but none replicates in enucleate cells with greater efficiency than VSV (unpublished data).

Unlike VSV, the initiation of an infection with influenza virus appears to be completely dependent on nuclear functions. Thus, in enucleated cells, under experimental conditions which in normal cells allow the synthesis of all of the previously identified influenza virus-specific polypeptides, none of the influenza virus gene products can be detected. In this respect these observations confirm directly the results of previous investigations of the effects of actinomycin D and UV-irradiation on influenza virus replication (1, 8; J. J. Skehel, *Virology*, in press). However, they do not at this stage give any indications of which cellular functions are specifically required by the influenza viruses, but additional investigations using this system, particularly those involved with virus nucleic acid synthesis, may provide

information on this point. It is also likely that the procedures described for the enucleation of cells after the initial stages of infection will be useful in attempts to elucidate the role of the nucleus in virus assembly and the significance of the accumulation of virus-specific components in nuclei during infection.

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