JOURNAL OF VIROLOGY, Jan. 1969, p. 8-16

Vol. 3, No. 1
Printed in U.S.A.

Formation of Viral Ribonucleic Acid and Virus in Cells that are Permissive or Nonpermissive for Murine Encephalomyelitis Virus (GDVII)[1]

LAWRENCE S. STURMAN[2] AND IGOR TAMM
The Rockefeller University, New York, New York 10021

Received for publication 24 July 1968

GDVII virus growth in BHK-21 cells, a permissive host for the virus, resembled productive infections with other picornaviruses. Virus yields ranged from 100 to 600 plaque-forming units (PFU)/cell. Virus replication in HeLa cells, a nonpermissive host for GDVII virus, was characterized by virus yields of only 0.1 to 5 PFU/cell. Similar low yields of virus have been obtained from HeLa cells at all multiplicities of input up to 6,000 per cell. The progeny particles from HeLa cells were, like the infecting particles, restricted in the HeLa cell host. Despite the great difference in final yields of virus from BHK-21 and HeLa cells, the times when maximal yields were reached were similar. GDVII virus stock grown in BHK-21 cells was designated HeLa$^-$. A variant of GDVII virus which is capable of extensive growth in HeLa cells was obtained. This variant, designated HeLa$^+$ GDVII virus, was passaged serially in HeLa cells. Virus yields of 50 to 150 infective virus particles per cell were obtained from infection of HeLa cells with HeLa$^+$ GDVII virus. The major species of HeLa$^+$ virus-specific ribonucleic acid (RNA) produced was single stranded and sedimented with an *S* value of 35*S*. The rate of accumulation of HeLa$^+$ virus-specific RNA in HeLa cell cultures was about four times that of HeLa$^-$ RNA. The amount of virus-specific HeLa$^+$ RNA formed in HeLa cells was several-fold greater than that of HeLa$^-$ RNA. With HeLa$^-$ parent GDVII virus undergoing productive replication in BHK-21 cells or abortive replication in HeLa cells, the major species of virus-specific RNA produced was single stranded and sedimented with an approximate *S* value of 35*S*. The amount of HeLa$^-$ virus-specific RNA extracted from BHK-21 cells was several-fold greater than the amount obtained from HeLa cells.

We reported in 1966 that GDVII virus, a murine encephalomyelitis virus, undergoes a complete or abortive replication cycle in tissue culture, depending on the cell type used (10). GDVII virus grows to high titer in BHK-21 and L cell lines, but in several other cell types, including HeLa cells, its multiplication is restricted and virus yields are only about 1% of those obtained in permissive hosts. Similar observations have subsequently been made with two other picornaviruses (3).

Restricted cycles of GDVII virus growth are associated with cytopathological changes (10). In HeLa cells, extensive cell destruction occurs within 12 to 18 hr after infection. The extent of cell damage is directly related to the amount of virus inoculated. The cytopathological changes associated with abortive multiplication appear to depend on the expression of a genetic function of the input virus.

In the present communication, we describe the kinetics of synthesis of viral ribonucleic acid (RNA) and of production of infective virus in permissive and nonpermissive cell hosts infected with GDVII virus.

MATERIALS AND METHODS

Cell cultures and media. The origin of baby hamster kidney (BHK-21) and HeLa cells and the growth media for these cells were described previously (10). The medium for the growth of virus in BHK-21 cell cultures was a modified reinforced Eagle's medium (10) supplemented with 10% tryptose phosphate broth and in some instances with 10% unheated calf

[1] A summary of this work was presented at the 68th Annual Meeting of the American Society for Microbiology (Bacteriol. Proc., p. 163, 1968).

[2] Present address: National Institute of Allergy and Infectious Diseases, Bethesda, Md. 20014.

serum. The medium for the growth of virus in HeLa cell cultures consisted of reinforced Eagle's medium (1) supplemented with 10% tryptose phosphate broth.

Virus stocks. GDVII virus, supplied as a mouse brain suspension by Max Theiler, was propagated several times by intracerebral inoculation of Swiss mice. It was then used to infect cultures of BHK-21 cells and produced high yields in such cultures. The BHK-21 cell-derived virus stock employed in the present work represents the fifth BHK-21 cell passage following triple plaque purification (which was carried out after 18 consecutive passages in BHK-21 cells). The virus stock was prepared by infecting confluent BHK-21 cell monolayers in 100-mm plastic tissue culture dishes as follows. Monolayers were washed once with phosphate-buffered saline (PBS; 10) before infection. The inoculum, 1.5 ml per dish, consisted of undiluted GDVII virus of high titer, representing pooled frozen and thawed infected cells and medium. After adsorption for 90 min, 9 ml of modified reinforced Eagle's medium, supplemented with 10% calf serum and 10% tryptose phosphate broth, was added to each culture without removing the inoculum. Released virus was harvested 22 hr after infection, clarified by centrifugation, frozen in 2- to 30-ml amounts in an alcohol-dry ice bath, and stored at −25 or −60 C. The titer of this virus stock in BHK-21 cell cultures was 5×10^8 plaque-forming units (PFU)/ml. For some experiments, we used virus which had not been plaque purified.

GDVII virus, grown either in mouse brain or in BHK-21 cells, undergoes a restricted cycle of multiplication in HeLa cells, with production of cytopathic effects but very little infective virus. HeLa cells are, therefore, considered to be a nonpermissive host for GDVII virus, and the stock virus grown in BHK-21 cells was designated HeLa$^-$.

A variant of GDVII virus capable of extensive growth in HeLa cells was obtained by alternating passage in HeLa and BHK-21 cells. GDVII virus from the 14th consecutive passage in BHK-21 cells without plaque purification was submitted to the following passages: two in HeLa, one in BHK-21, one in HeLa, one in BHK-21, and two in HeLa cells. Cells and medium from each passage were combined, frozen and thawed three times, clarified by centrifugation, and used undiluted as inoculum for the succeeding passage. Progressive cytopathic effects were observed on the last passage in HeLa cells, and hemagglutination titrations indicated considerable multiplication of virus. The virus capable of growing in HeLa cells was designated HeLa$^+$ and was passaged serially in HeLa cells. The HeLa$^+$ virus stock used in the present studies represents the 18th consecutive HeLa cell passage. The stock was prepared by infecting confluent HeLa cell monolayers in 100-mm plastic tissue culture dishes. Monolayers were washed once with PBS before infection. The inoculum, 1.5 ml per dish, consisted of undiluted released virus of high titer. After adsorption for 60 min, 8 ml of reinforced Eagle's medium supplemented with 10% tryptose phosphate broth was added to each culture without removing the inoculum. Released virus was harvested 23 hr after infection, clarified by centrifugation, frozen in 2- to 30-ml portions in an alcohol-dry ice bath, and stored at −25 C. The titer of this virus stock in HeLa cell cultures was 10^8 TCID/ml.

HeLa$^+$ virus is neutralized by antiserum prepared against mouse brain-derived or BHK-21 cell-derived HeLa$^-$ GDVII virus.

Intracerebral inoculation of mice with HeLa$^+$ GDVII virus causes a disease indistinguishable from that produced by BHK-21 cell-derived HeLa$^-$ GDVII virus. No quantitive comparisons have been made, however, between the two virus strains with respect to virulence for mice.

Virus assays. The technique employed for plaque assay of HeLa$^-$ GDVII virus in BHK-21 cell monolayers was described previously (10). The 50% infectivity end point titrations of HeLa$^+$ GDVII virus were performed on monolayers of HeLa cells grown in 35-mm plastic tissue culture dishes. Serial 0.7 log unit dilutions of the virus were prepared. Groups of six cultures were infected with suitably diluted virus. Cultures were examined on the third to fifth days after infection, and 50% tissue culture infectivity end points ($TCID_{50}$) were calculated by the method of Reed and Muench (8). The 50% infective end points were converted to infective units by multiplying by 0.69.

Hemagglutination titrations were performed as described previously (10).

Extraction and sedimentation of virus-specific RNA. The medium was removed from labeled, infected BHK-21 or HeLa cell monolayers, and the cell sheets were washed once with PBS at 4 C. The cells were scraped off the culture dish and suspended in 0.02 M phosphate buffer, *p*H 7.2. Sodium dodecyl sulfate was added to a final concentration of 0.5% and ethylenediaminetetraacetic acid (EDTA) was added to a final concentration 0.01 M. The cells were extracted twice at 25 C with equal volumes of buffer-saturated phenol. Addition of a few drops of isoamyl alcohol after the first extraction aided in the separation of the phenol and aqueous phases. Residual phenol was removed by three extractions with ether, and this was followed by passage of nitrogen gas through the solution until the odor of ether was no longer detected. Volumes (1 ml) of such RNA extracts were layered at 4 C on 29-ml, linear 15 to 30% sucrose gradients preformed by the procedure described by Britten and Roberts (2) in 0.15 M NaCl, 0.015 M sodium citrate, and 0.001 M EDTA. The gradients were centrifuged at 4 C in the SW25 rotor of a Spinco model L2 ultracentrifuge for 19 hr at 58,600 × *g*. At the end of the run, fractions of equal volume were collected from the top of the gradient by displacement with a 38% sucrose solution pumped through the bottom of the tube. Continuous recordings of optical density were obtained by passage of the effluent through an Isco model UA-2 flow cell. Sedimentation coefficients of various types of virus-specific RNA were calculated by the method described by Martin and Ames (7).

Radiochemical counting procedures. Radioactivity was determined in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers

Grove, Ill.). The scintillation fluid was a toluene-Liquifluor mixture which contained 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter. Scintillation fluid (10 ml) was added to each sample.

For analysis of sucrose gradient sedimentation profiles of ^{3}H-uridine-labeled virus-specific RNA, samples from the gradients were prepared by precipitation with 5% trichloroacetic acid, and precipitates were collected on Whatman GF/C fiber glass filter pads (2.4 cm). Bovine serum albumin, 50 or 100 μg, was added as carrier to each sample. Each precipitate was collected on a filter pad with suction and washed twice with 2- to 3-ml portions of 5% trichloroacetic acid. The glass-fiber filter pads were dried in an oven at 70 C. For measurement of ribonuclease-resistant RNA, samples were treated with 100 μg of pancreatic ribonuclease (Worthington, Biochemical Corp., Freehold, N.J.; 3 × recrystallized) per ml for 30 min at room temperature in 0.3 M NaCl before precipitation with trichloroacetic acid.

For measurement of radioactive ^{3}H-uridine incorporation into virus-specific RNA by cells grown on glass cover slips, the cover slips were fixed in cold acetic acid-ethyl alcohol (1:3) for 15 min and then were placed in cold 70% ethyl alcohol for at least 1 hr. Acid-soluble ^{3}H-uridine was removed by extraction with cold 5% perchloric acid for 30 min (two changes), followed by thorough washing in demineralized water (five changes). The cover slips were passed through ether-ethyl alcohol (1:1) and anhydrous ether and were dried in an oven at 70 C.

Autoradiography. For autoradiography, cover slips were mounted with Permount, cell surface side up, on uncoated glass slides and were coated by immersion in Kodak NTB2 emulsion. When dry, the slides were placed in black plastic lightproof boxes, sealed, and stored at 4 C for 24 hr. The slides were then developed and stained with a modified Giemsa stain (9). Grains were counted with phase contrast illumination at a magnification of 800×. Grain counts are based on examination of 100 cells on each cover slip in randomly selected fields.

Chemicals. Actinomycin D was obtained from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J. ^{3}H-uridine, specific activity 15 to 30 c/mmole, was purchased from Nuclear-Chicago Corp., Des Plaines, Ill. Liquifluor was purchased from Pilot Chemicals, Inc., Watertown, Mass.

RESULTS

Growth kinetics of HeLa$^-$ GDVII in BHK-21 cells and the rate of viral RNA synthesis. The rate of synthesis of HeLa$^-$ GDVII virus-specific RNA and the kinetics of formation of infective and hemagglutinating particles in the productive infection of BHK-21 cells were determined.

BHK-21 cell monolayers were washed once with PBS and were inoculated with plaque-purified HeLa$^-$ GDVII virus at a multiplicity of 330 PFU/cell. After adsorption of virus for 1 hr at 37 C, the monolayers were washed and incubated with fresh medium containing 1 μg of actinomycin D per ml. ^{3}H-uridine, 10 μc/ml, was added for a 15-min pulse at hourly intervals beginning 1.75 hr after infection, and incorporation into RNA was determined. Production of infective and hemagglutinating virus was measured in similarly infected cultures which did not receive actinomycin D. These cultures were also used to follow the development of virus-induced cytopathological changes (Fig. 1).

Synthesis of GDVII virus-specific RNA became detectable approximately 3 hr after infection. The rate of virus-specific RNA synthesis reached a maximum at 7 hr and then gradually declined. Newly synthesized viral antigen became detectable by fluorescent antibody staining in the cytoplasm of infected cells 5 hr after infection. Virus production was first detected 5 hr after inoculation. During the exponential increase

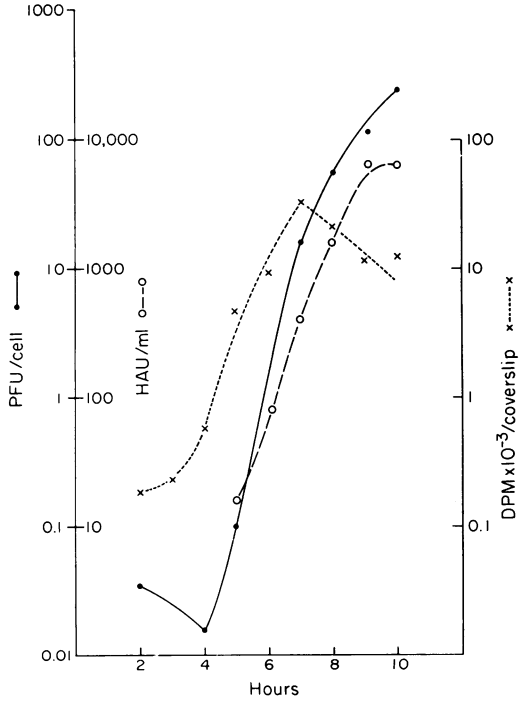

FIG. 1. *Growth of HeLa$^-$ GDVII virus in BHK-21 cells and rate of synthesis of viral RNA. Input multiplicity: 330 PFU/cell. In determinations of virus-specific RNA synthesis, 1 μg of actinomycin D per ml was added after a 1-hr adsorption period, and ^{3}H-uridine, 10 μc/ml, was added for a 15-min pulse at hourly intervals beginning 1.75 hr after infection. Production of infective and hemagglutinating virus was measured in similarly infected cultures which did not receive actinomycin D. Yields of infective and hemagglutinating virus represent the sums of virus in cells and medium.*

phase, the amount of virus doubled every 9 to 12 min. Maximal yields of virus were reached 12 to 16 hr after inoculation, and they ranged from 100 to 600 PFU/cell. Thus, large amounts of infective and hemagglutinating virus were produced at a time when the rate of viral RNA synthesis was declining. Viral cytopathic effects were first detected at 8 hr, and more than 90% of the cells became rounded and detached from the surface of the culture dish by 14 hr.

Growth kinetics of HeLa⁻ GDVII virus in HeLa cells. The kinetics of formation of infective HeLa⁻ virus were determined in the abortive infection of nonpermissive HeLa cells.

HeLa cell monolayers were washed once with PBS and were inoculated with plaque-purified BHK-21 cell-derived GDVII virus at a multiplicity of 680 PFU/cell. After adsorption for 1 hr at 37 C, the monolayers were washed and incubated with fresh medium containing anti-GDVII immune serum. The medium containing antiserum was removed after 30 min, the cell monolayers were washed twice with PBS, and fresh, reinforced Eagle's medium supplemented with 10% tryptose phosphate broth was added. At intervals, the cells were scraped off, and cells and media from two plates were collected, pooled, and assayed for infective virus by plaque formation in BHK-21 cell cultures.

The results are shown in Fig. 2. For comparison, the kinetics of GDVII virus production in BHK-21 cells are also shown in the same figure. In this experiment, maximal yields of 0.2 to 0.3 PFU per cell in HeLa cell cultures were obtained by 12 to 16 hr. Viral cytopathic effects were first detected at 12 hr, and more than 95% of the cells were destroyed by 16 to 20 hr after infection.

Similar yields of HeLa⁻ GDVII virus, i.e., 0.1 to 5 PFU per cell, have been obtained at all multiplicities of input up to 6,000 per cell. As can be seen by comparison of the two growth curves, despite the great difference in final yields of GDVII virus obtained from BHK-21 and HeLa cells, the times when final yields were reached were similar.

Isolation of HeLa⁺ GDVII virus and growth kinetics in HeLa cells. To determine the ways in which the replication of HeLa⁻ GDVII virus may be restricted in HeLa cells, we thought it would be useful to have a variant of GDVII virus which is not restricted in this cell host. Such a variant was obtained after a series of alternating passages of GDVII virus in HeLa and BHK-21 cells. This variant, designated HeLa⁺ GDVII virus, was passaged serially in HeLa cells. Infection of HeLa cells with HeLa⁺ virus yielded 50 to 150 infective virus particles per cell. These yields are somewhat lower than those of HeLa⁻ GDVII virus in BHK-21 cells. Serial propagation of plaque-purified HeLa⁺ virus in BHK-21 cell cultures yielded virus which exhibits reduced ability for multiplication in HeLa cells.

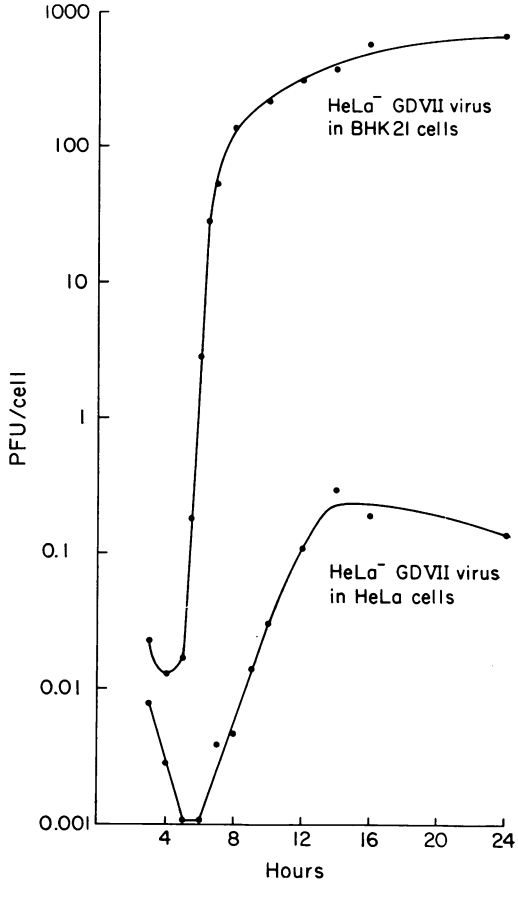

FIG. 2. *Growth of HeLa⁻ GDVII virus in HeLa and BHK-21 cells. Input multiplicity: 680 PFU/HeLa cell; 20 PFU/BHK-21 cell. Yields represent the sums of virus in cells and medium.*

The growth curve of HeLa⁺ GDVII virus in HeLa cells is shown in Fig. 3. In this experiment, the input multiplicity was 15 infective particles per cell. A portion of the growth curve of HeLa⁻ GDVII virus in the same cell type is shown for comparison. Since antiserum was not employed to reduce the level of residual HeLa⁺ virus remaining from the inoculum, the background level was higher than in the previous experiments. In this experiment, HeLa⁺ GDVII virus production was detected 6 hr after infection, but it probably began earlier. By 12 hr, 100 infective particles of HeLa⁺ GDVII virus had been produced per cell. By comparison, the final yield of HeLa⁻ GDVII virus in HeLa cells was less than 1% of this value.

The growth and cytopathic effects of HeLa⁻

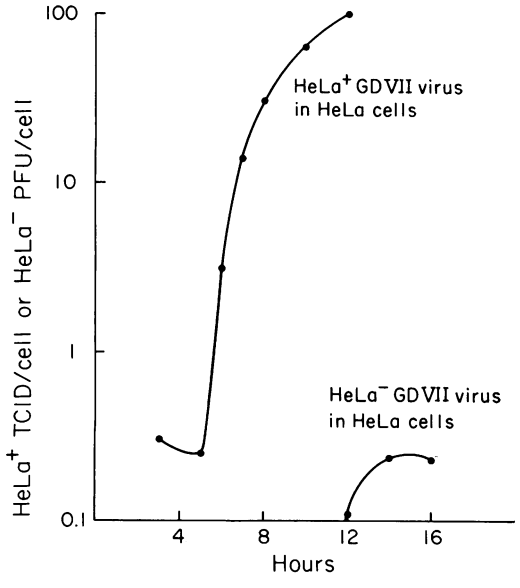

FIG. 3. *Growth of HeLa$^+$ and HeLa$^-$ GDVII virus in HeLa cells. Input multiplicity: 15 HeLa$^+$ TCID/cell; 680 HeLa$^-$ PFU/cell. The portion of the growth curve of HeLa$^-$ GDVII virus in HeLa cells is taken from Fig. 2. Yields represent the sums of virus in cells and medium.*

and HeLa$^+$ GDVII viruses in HeLa cells are reviewed in Table 1. In HeLa cells, the yield of HeLa$^+$ virus was 30- to 500-fold greater than that of HeLa$^-$ virus. HeLa$^+$ and HeLa$^-$ viruses both caused marked cytopathological changes in HeLa cells, but such changes developed more slowly after infection with HeLa$^-$ virus. HeLa$^+$ virus could be serially propagated in HeLa cells, whereas the progeny particles of HeLa$^-$ virus were, like the infecting particles, restricted in this host.

Infectivity assays of HeLa$^-$ and HeLa$^+$ GDVII viruses. Procedures and results of infectivity assays of HeLa$^-$ and HeLa$^+$ viruses are summarized in Table 2.

With HeLa$^-$ virus, the plaque and infectivity end point assays in BHK-21 cells were in substantial agreement. In HeLa cells, neither assay was possible with HeLa$^-$ virus.

With HeLa$^+$ virus, the infectivity end point assay in HeLa cells gave 10- to 50-fold higher values than plaque assay in either HeLa or BHK-21 cells.

Hemagglutination by HeLa$^-$ and HeLa$^+$ viruses. Both HeLa$^-$ and HeLa$^+$ GDVII viruses agglutinated human red blood cells. The ratio of infectivity to hemagglutinating activity for HeLa$^-$ GDVII virus was 10^4 to 2×10^4 PFU (measured in BHK-21 cells); for HeLa$^+$ GDVII virus, this ratio was 10^6 TCID (measured in HeLa cells). The difference in the ratios for HeLa$^-$ and HeLa$^+$ GDVII virus may reflect a difference either in efficiency of hemagglutination or in infectivity assays or both.

Accumulation of GDVII virus-specific RNA in HeLa cells. The rates of accumulation of GDVII virus-specific RNA were investigated in HeLa cell cultures and in individual HeLa cells in cultures infected with HeLa$^-$ or HeLa$^+$ GDVII virus.

Overall rates of accumulation were determined as follows. Monolayers of HeLa cells grown on glass cover slips were infected at high multiplicities with either HeLa$^-$ or HeLa$^+$ GDVII virus. The accumulation of virus-specific RNA was measured by the amount of tritiated uridine incorporated into an acid-insoluble form in actinomycin-treated cultures. Tritiated uridine, 10 μc/ml, was added to groups of cultures hourly from 5 to 11 hr after infection, and four cover slips were harvested 3 hr after each addition. Moreover, after addition of tritiated uridine at 5 hr after infection, cover slips were also harvested 1 and 2 hr later. Cumulative curves of synthesis of virus-specific HeLa$^+$ and HeLa$^-$ RNA in HeLa cells are shown in Fig. 4. The rates of accumulation of HeLa$^+$ and HeLa$^-$ virus RNA were nearly constant after the 6th and 7th hr, respectively, but the rate of accumulation of HeLa$^-$ virus-specific RNA was approximately one-fourth of that of HeLa$^+$ RNA. Occurrence of viral cytopathic effects prevented the observation of HeLa$^+$ virus-infected cells beyond 9 to 10 hr after infection; however, in HeLa$^-$ virus-infected cultures there was no significant loss of cells until much later in the growth cycle and the increase in HeLa$^-$ GDVII RNA remained linear to the 14th hr after infection.

For a study of virus-specific RNA in individual cells, duplicate cover slips from the same experiment were prepared for autoradiography. The interval after infection which was selected for comparison was that 3-hr period during which the

TABLE 1. *Interaction of HeLa$^-$ and HeLa$^+$ GDVII viruses with HeLa cells*

Virus effect in HeLa cells	Virus strain	
	HeLa$^-$[a]	HeLa$^+$[b]
Maximal yield	0.1–5 PFU/cell	50–150 TCID/cell
Cytopathic effects	3–4+	4+
Multiple cycles of replication	No	Yes
Plaque formation	No	Yes

[a] Grown in BHK-21 cells.
[b] Grown in HeLa cells.

TABLE 2. *Infectivity assays of HeLa⁻ and HeLa⁺ GDVII viruses*

GDVII virus strain	BHK-21 cells			HeLa cells		
	Plaque size	Relative sensitivity of plaque assay	Relative sensitivity of 50% infectivity end point assay	Plaque size	Relative sensitivity of plaque assay	Relative sensitivity of 50% infectivity end point assay
HeLa⁻	3–5 mm at 5 days	1[a]	0.5–1			$<10^{-4}$
HeLa⁺	0.5–2 mm at 5 days	0.02–0.1	Not done	0.5–2 mm at 5 days	0.02–0.1	1[b]

[a] With HeLa⁻ virus, plaque assay in BHK-21 cells gives the highest titers, and therefore the sensitivity of this titration procedure with HeLa⁻ virus is assigned the value 1.

[b] With HeLa⁺ virus, end point titration in HeLa cells gives the highest values.

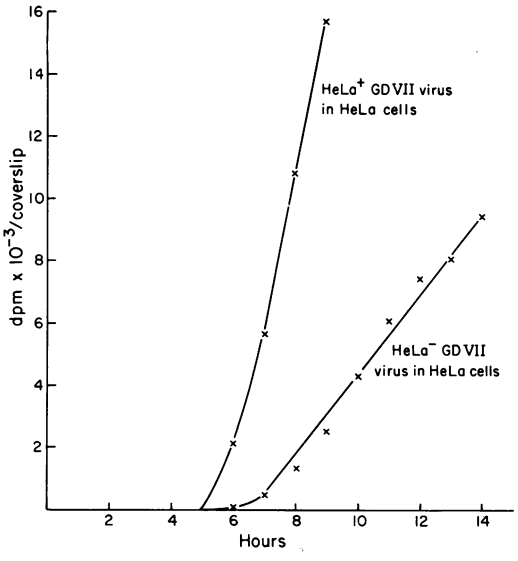

FIG. 4. *Accumulation of HeLa⁻ and HeLa⁺ GDVII virus-specific RNA in HeLa cells. Monolayers of HeLa cells grown on glass cover slips were infected with either HeLa⁻ or HeLa⁺ GDVII virus at multiplicities of 700 and 100, respectively. At hourly intervals, 4 to 10 hr after infection, 5 μg of actinomycin D per ml was added to groups of infected cultures. One hour later, ³H-uridine was introduced to a final concentration of 10 μc/ml. Uridine incorporation was continued for 3 hr. In addition, cover slips which received ³H-uridine at 5 hr after infection were also harvested 1 and 2 hr later. The accumulation of virus-specific RNA was measured by the amount of ³H-uridine incorporated into an acid-insoluble form. Since exposure to actinomycin D caused deterioration of HeLa cells in 5 to 6 hr, accumulation of virus-specific RNA was reconstructed from incorporation of ³H-uridine during overlapping 3-hr periods. The design of this experiment also permitted autoradiographic analysis of virus-infected cultures exposed to ³H-uridine during the 3-hr period when synthesis of the greatest amount of virus-specific RNA occurred (Fig. 5).*

most rapid accumulation of virus-specific RNA was obtained with each virus, namely, 9 to 12 hr after infection for HeLa⁻ and 6 to 9 hr after infection for HeLa⁺ GDVII virus.

Grain counts on infected cultures were performed on 100 cells on each cover slip. The results are illustrated in Fig. 5, in the form of bar graphs showing the numbers of cells with increasing numbers of grains. The background in uninfected cultures was between two and three grains per cell. Cells with more than 100 grains are grouped together at the extreme right.

One-third of the cells infected with HeLa⁻ GDVII virus showed less than 10 grains per cell, and another one-third of the cells contained more than 30 grains per cell. In contrast, less than 5% of the cells in the culture infected with HeLa⁺ GDVII virus contained fewer than 10 grains per cell, whereas at least 30 grains occurred in each of more than two-thirds of the cells. It is apparent from this distribution of grains that virus-specific RNA accumulates at a slower rate in cells infected with HeLa⁻ virus than it does in cells infected with HeLa⁺ GDVII virus.

One measure of the maximal rate of accumulation of virus-specific RNA per cell, based on autoradiographic analysis, is the mean grain number for cells containing more than 10 grains. The lower limit of 10 is three standard deviations from the mean number of grains in uninfected cells. Such an estimate indicated that the maximal rate of accumulation of HeLa⁻ virus-specific RNA is at least 40% lower than that of HeLa⁺ RNA. Some of the cells containing fewer than 10 grains probably also produced HeLa⁻ virus-specific RNA. For this reason, the estimate of the maximal rate of accumulation of virus-specific RNA in HeLa⁻ virus-infected cells was probably too high.

Species of GDVII virus-specific RNA produced in BHK-21 and HeLa cells. To determine whether the same species of GDVII virus-specific RNA are produced during infection of permissive and nonpermissive cell hosts, the sedimentation pattern of HeLa⁻ virus-specific RNA produced in BHK-21 cells was compared with the distributions of HeLa⁻ and HeLa⁺ virus-specific RNA produced in HeLa cells. BHK-21 and HeLa cell cultures were infected at high multiplicities with

the same preparation of HeLa$^-$ GDVII virus, and duplicate HeLa cell cultures were infected at a high multiplicity with HeLa$^+$ GDVII virus. The cell cultures were treated with actinomycin D, 5 μg per ml, 1 hr before the addition of tritiated uridine to a final concentration of 10 μc per ml. BHK-21 cells infected with HeLa$^-$ GDVII virus were labeled with tritiated uridine from 6 to 9 hr after infection. HeLa cells infected with HeLa$^-$ virus were labeled with tritiated uridine from 7 to 11 hr after infection, and duplicate cultures infected with HeLa$^+$ GDVII virus were labeled from 6 to 10 hr after infection. RNA was extracted from suspensions of whole cells by phenol and the extracts were sedimented through 15 to 30% sucrose gradients (Fig. 6).

In both permissive and nonpermissive cell types, the major species of HeLa$^-$ virus-specific RNA produced sedimented with an approximate *S* value of 34*S* to 35*S* (left and center diagrams in Fig. 6). The virus-specific 35*S* RNA was sensitive to digestion with pancreatic ribonuclease and is probably single stranded. In analogy with results obtained with poliovirus, this RNA species probably represents the viral RNA. In both cell types, labeled uridine was also incorporated into species of RNA which sediment in a broad region of the gradient from 14*S* to 28*S*. A portion of this RNA was relatively resistant to pancreatic ribonuclease digestion and may be double stranded.

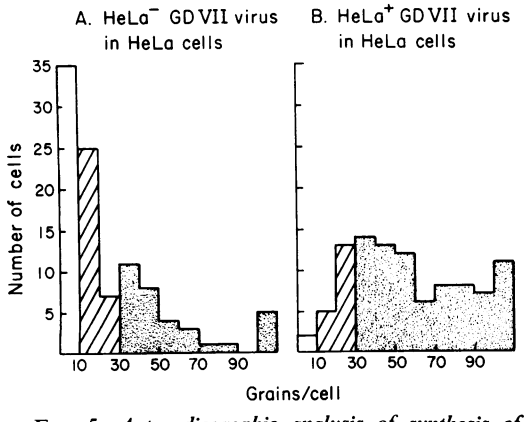

FIG. 5. *Autoradiographic analysis of synthesis of virus-specific RNA in HeLa cells infected with HeLa$^-$ or HeLa$^+$ GDVII virus. Duplicate cover slips from the experiment described in Fig. 4 were prepared for autoradiography. The interval after infection which was selected for comparison was that 3-hr period during which maximal accumulation of virus-specific RNA was obtained with each virus, namely 9 to 12 hr after infection with HeLa$^-$ virus and 6 to 9 hr after infection with HeLa$^+$ GDVII virus. Grain counts on infected cultures were performed on 100 cells on each cover slip in randomly selected fields. The background in uninfected cultures, based on counts on 200 cells, was between 2 and 3 grains per cell. The height of each bar corresponds to the number of cells with grain counts within multiples of 10 grains. Cells with more than 100 grains are grouped together at the extreme right.*

The sedimentation pattern of labeled HeLa$^+$ RNA from HeLa cells revealed a major peak sedimenting with an *S* value of approximately 35*S* and a shoulder extending to about 20*S* (diagram on right in Fig. 6). There was a smaller peak of labeled material in the region of 14*S* to 20*S*. Much of the RNA in the smaller peak was resistant to digestion with pancreatic ribonuclease, whereas the RNA in the 35*S* peak was ribonuclease-sensitive.

Since the three gradients contained approximately equal quantities of cellular RNA, as measured by optical density at 260 nm, and comparable periods in the growth cycles were selected for study, the results indicated that the production of HeLa$^-$ GDVII viral RNA is restricted in the nonpermissive HeLa cell host.

DISCUSSION

The host cell range of GDVII virus was established previously (10). The murine picornavirus GDVII undergoes a restricted cycle of growth in some cell types of mouse, sheep, monkey, or human origin. GDVII virus causes cytopathic effects in a nonpermissive host such as the HeLa cell, although little virus is produced. Because of restricted replication in HeLa cells, we designated this virus HeLa$^-$. The same virus causes a productive infection in BHK-21 Syrian hamster kidney cells and certain other cell types of hamster or mouse origin. HeLa$^-$ GDVII virus growth in permissive hosts such as BHK-21 cells closely resembles productive infection with other picornaviruses; the virus yields range from 100 to 600 PFU/cell and cytopathological changes develop rapidly in the infected cells.

In the nonpermissive host, the HeLa cell, production of infective virus is characterized by the following features. The amount of infective HeLa$^-$ GDVII virus progeny produced is only 0.1 to 5 PFU/cell. Similar yields of virus have been obtained from HeLa cells at multiplicities of input up to 6,000 per cell. The progeny particles from HeLa cells are, like the infecting particles, restricted in the HeLa cell host. Despite the great difference in yields of HeLa$^-$ GDVII virus from HeLa and BHK-21 cells, the times when final yields are reached in nonpermissive or permissive cells are similar. The differences in the rate of development of viral cytopathic effects in the productive and restricted cycles of GDVII virus growth may reflect differences in rates of accumu-

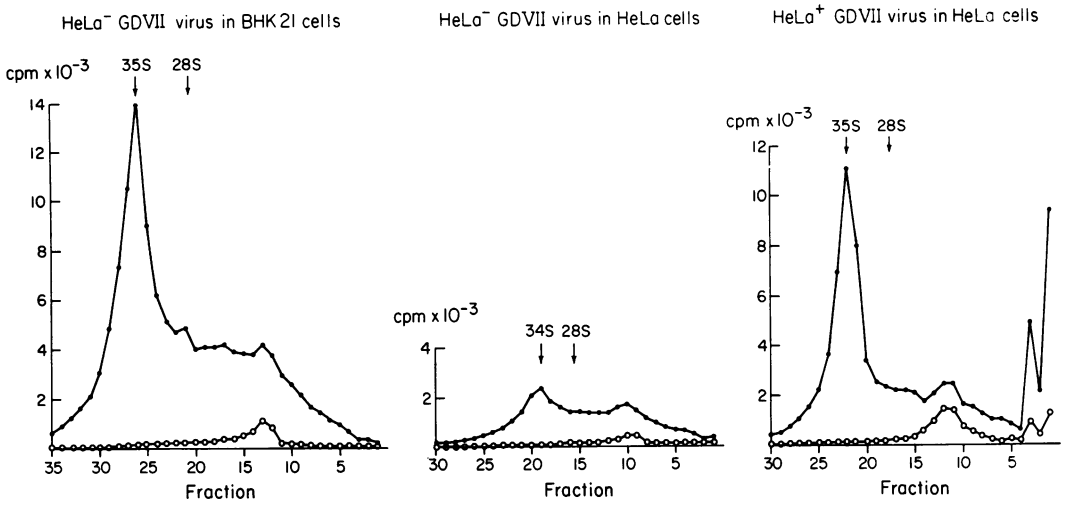

FIG. 6. *Sedimentation of ^{3}H-uridine-labeled virus-specific RNA from BHK-21 cells infected with HeLa⁻ virus and from HeLa cells infected with HeLa⁻ or HeLa⁺ virus. Cultures of BHK-21 and HeLa cells were infected with the same preparation of HeLa⁻ GDVII virus at multiplicities of 30 and 150, respectively. BHK-21 and HeLa cells were exposed to 5 μg of actinomycin D per ml 5 and 6 hr after infection, respectively. One hour later, tritiated uridine was added to a final concentration of 10 μc/ml. BHK-21 cells were exposed to the isotope from 6 to 9 hr and HeLa cells from 7 to 11 hr. Duplicate cultures of HeLa cells were infected with HeLa⁺ GDVII virus at a multiplicity of 30, treated with 5 μg of actinomycin D per ml beginning 5 hr after infection, and labeled with tritiated uridine from 6 to 10 hr after infection. RNA was extracted from suspensions of whole cells by phenol, and the extracts were sedimented through 15 to 30% sucrose gradients at 58,600 × g for 19 hr in an SW25 rotor of a Spinco model L2 centrifuge. The bottom of each gradient is at the left. The upper curves represent the acid-insoluble counts in one-half of each fraction. The lower curves represent the acid-insoluble counts remaining after pancreatic ribonuclease digestion of the remaining half of each fraction. The position of 28S ribosomal RNA is indicated by the arrow on the right in each diagram. The gradients contained approximately equal quantities of cellular RNA, as measured by optical density at 260 nm.*

lation of cytotoxic viral products in the two host cell types.

Restriction of the growth of GDVII virus in HeLa cells is not due to a general defect in the ability of the cells to support the multiplication of small RNA viruses. Other picornaviruses, such as poliovirus, multiply well in HeLa cells with production of several hundred infective particles per cell. Furthermore, a variant of GDVII virus which is capable of extensive growth in HeLa cells has been obtained. The variant has been designated HeLa⁺. Usually virus yields of 50 to 150 infective virus particles per cell are obtained from HeLa cells infected with HeLa⁺ GDVII virus.

The rate of accumulation of HeLa⁺ virus-specific RNA in HeLa cell cultures is about four times that of HeLa⁻ RNA. The amount of virus-specific RNA extracted from HeLa⁺ virus-infected HeLa cells per unit of cellular RNA is several-fold greater than that obtained from HeLa⁻ virus-infected HeLa cells under similar conditions. Thus, the restricted multiplication of HeLa⁻ GDVII virus in HeLa cells is associated with restricted synthesis of the viral RNA.

By sedimentation analysis on sucrose density gradients, HeLa⁻ GDVII virus-specific RNA synthesized in HeLa and BHK-21 cells and HeLa⁺ GDVII virus RNA synthesized in HeLa cells are qualitatively similar. In both the abortive infection in HeLa cells and the productive infection in BHK-21 cells, the major species of HeLa⁻ virus-specific RNA produced is single stranded and sediments with an approximate *S* value of 35*S*. However, the amount of labeled HeLa⁻ virus-specific RNA obtained from HeLa cells is several-fold lower than that from BHK-21 cells. The major species of HeLa⁺ virus-specific RNA produced in HeLa cells is also single stranded and sediments with an approximate *S* value of 35*S*.

Since our first report (10), it has been found that two other picornaviruses, mengovirus and bovine enterovirus 1, also cause abortive infections in several different animal cell hosts (3). Cells which support a productive infection with one of the two viruses may be nonpermissive for the other. One cell type which is a permissive host for both mengovirus and bovine enterovirus 1 is the HeLa cell, a nonpermissive host for GDVII virus. No variant of mengovirus or bovine entero-

virus 1 with extended host range (analogous to HeLa$^+$ GDVII virus) has been reported.

No qualitative differences have been discovered in the pattern of mengovirus-directed protein synthesis through analysis by acrylamide gel electrophoresis of extracts from productively or abortively infected cells (6). Though the total amount of viral proteins synthesized in nonpermissive cell hosts is reduced, the different proteins are synthesized in similar proportions in nonpermissive and permissive cells.

The evidence obtained in the present investigation indicates that host-dependent restriction of picornavirus growth is associated with restricted synthesis of the viral RNA. Reduced synthesis of viral RNA could well lead to reduced synthesis of virus-specific proteins, as observed in infection of nonpermissive cells with mengovirus (6). It should be emphasized, however, that RNA replication depends both on successful translation and transcription of the viral genome, and that these two processes are interdependent (5). Synthesis of viral RNA, which in picornaviruses is also the messenger RNA, depends on the synthesis of viral RNA polymerase. Reduction in the synthesis of the viral RNA polymerase would be expected to lead to reduced synthesis of viral RNA. Further work is clearly needed to elucidate the nature of the primary defect in picornavirus replication in nonpermissive cells. We do know, however, that the restricted infection of HeLa cells with HeLa$^-$ GDVII virus is not one in which normal amounts of RNA are made while production of infective virus is curtailed. Rather, the evidence which we obtained indicates that the production of HeLa$^-$ viral RNA itself is restricted.

Although the cytopathological changes indicate that all cells in a culture can be infected under conditions of restricted growth of virus in nonpermissive cells, the possibility exists that an early step, such as uncoating, is delayed or incompletely executed. Our results could, at least in part, be explained on the basis of delayed or restricted initiation of messenger and replicative functions by the RNA of the infecting particles. Host-dependent differences in the rate or efficiency of uncoating have been reported previously for poliovirus (4). Another possibility is that input or progeny viral RNA or the replicating viral RNA may not interact properly with the membranous structures with which picornavirus-directed biosynthesis is associated in the infected cell. This, too, could result in limited synthesis of viral RNA as well as proteins.

Restriction in the production of infective virus is more than 10-fold greater than restriction in the synthesis of viral RNA. If there are strict spatial and temporal requirements for efficient encapsidation of viral RNA, even a moderate restriction in the total amount of RNA may lead to a very marked reduction in the formation of infective virus. On the other hand, limited availability of capsid proteins could also be a factor in the reduced production of HeLa$^-$ virus in HeLa cells.

In a previous communication (10), we discussed some of the known mechanisms that determine whether a particular phage is capable of replication in a particular bacterial cell to which it can adsorb. These mechanisms, which involve host-controled modification of nucleic acid and informational suppression, have not yet been reported for animal viruses, and there is at present no indication whether they may provide a biochemical basis for the specific host-dependent viariablity in the growth of picornaviruses.

Although, in general, different types of cells from the same species behave in the same way, it is of particular interest that two kinds of cells of mouse origin respond differently to GDVII virus infection. The same observation has been made with mengovirus which also undergoes productive or abortive infection in different cell types of mouse origin. This indicates that, depending on limited changes in the genome of the host cell, the ability of cells to support the replication of viruses may vary.

ACKNOWLEDGMENTS

The excellent technical assistance of Toyo Kikuchi and Barbara Nozick is gratefully acknowledged.

This investigation was supported by research grant AI-03445 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. Virology **26**:100–113.
2. Britten, R. J., and R. B. Roberts. 1960. High-resolution density gradient sedimentation analysis. Science **131**:32–33.
3. Buck, C. A., G. A. Granger, M. W. Taylor, and J. J. Holland. 1967. Efficient, inefficient, and abortive infection of different mammalian cells by small RNA viruses. Virology **33**:36–46.
4. Darnell, J. E., and T. K. Sawyer. 1960. The basis for variation in susceptibility to poliovirus in HeLa cells. Virology **11**:665–675.
5. Eggers, H. J., D. Baltimore, and I. Tamm. 1963. The relation of protein synthesis to formation of poliovirus RNA polymerase. Virology **21**:281–283.
6. Holland, J. J. 1968. Virus-directed protein synthesis in different animal and human cells. Science **160**:1346–1348.
7. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372–1379.
8. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
9. Schmid, W. 1963. DNA replication patterns of human chromosomes. Cytogenetics **2**:175–193.
10. Sturman, L. S., and I. Tamm. 1966. Host dependence of GDVII virus: complete or abortive multiplication in various cell types. J. Immunol. **97**:885–896.