JOURNAL OF VIROLOGY, Apr. 1975, p. 872-881
Vol. 15, No. 4

Printed in U.S.A.

Host-Dependent Restriction of Mengovirus Replication

III. Effect of Host Restriction on Late Viral RNA Synthesis and Viral Maturation

SUZANNE O. PRATHER[1] AND MILTON W. TAYLOR*

Microbiology Department, Indiana University, Bloomington, Indiana 47401

Received for publication 15 October 1974

Restricted mengovirus replication in Madin-Darby bovine kidney (MDBK) cells is characterized by a 400-fold reduction in infectious virus yield and a 40-fold increase in the production of noninfectious virus. Using conditions which insure that all MDBK cells are infected, virus-specific RNA and protein synthesis were measured in the restrictive host and in a permissive host for mengovirus, HeLa cells. Labeling kinetics and sucrose gradient analysis of mengovirus-specific RNA from MDBK cells show a reduction of 10-fold in virion RNA, 5-fold in double-stranded RNA, and 12.5-fold in single-stranded RNA. The viral RNA biosynthetic processes which occur late in the replicative cycle and result in the production of 90% of the single-stranded viral RNA that is packaged into capsid proteins in the permissive host are absent in restrictive MDBK cells. Viral protein synthesis as measured by labeled viral-specific polysome is decreased, and there is an accumulation of 80*S* subviral particles in the restricted host. It is suggested that restriction may act at a number of stages of viral replication and maturation.

Buck et al. (4) and Wall and Taylor (23, 24) have reported that the yield of mengovirus progeny depends on the host in which this virus is propagated. Productively infected L cells, HeLa cells, and Erlich ascites tumor cells produced more than 1,000 infectious mengovirus per cell, whereas a nonpermissive host, Madin-Darby bovine kidney (MDBK) cells, produces only five progeny mengovirus per cell (S. O. Prather, Ph.D. thesis, Indiana Univ., Bloomington, Ind., 1974; 4, 23). The mengovirus restriction phenomenon observed in MDBK cells does not appear to result from an absence of mengo-specific receptor sites on the cell surface, as the infective process proceeded for the first 4 h of the viral replicative cycle in a normal fashion (4, 23, 24). Additionally, mengovirus-infected MDBK cells show a pattern of cytopathogenicity similar to productively infected cells (23).

Wall and Taylor (23, 24) investigated the restricted replication of mengovirus in MDBK cells, with particular attention to the synthesis and accumulation of virus-specific RNA in the nonpermissive host when compared with the same in permissive Erlich ascites tumor and L cells. They reported that radioactive uridine accumulation into acid-insoluble material ceased prematurely at 4 h after infection in the inefficiently infected MDBK cells (25), and that the amount of mengovirus-specific RNA was about fourfold less than that accumulated in productively infected L cells (24). At early times after infection the same amounts of mengovirus replicative intermediate RNA, replicative-form RNA, and single-stranded RNA were synthesized in both L cells and MDBK cells (24), but by 5.5 h after infection the rate of synthesis of these three species of viral RNA was considerably diminished in mengovirus-infected MDBK cells, with single-stranded RNA being depressed about fourfold. However, pulse-labeling experiments utilizing a 10-min pulse of [^{3}H]uridine in both MDBK and L cells suggested that viral RNA was synthesized at the same rate in both permissive and nonpermissive hosts. Thus, the restrictive event could be explained as due to degradation of newly formed RNA in the restricted host. Since the rates of synthesis of virus-specific RNA were the same in both hosts, gel analyses of viral RNA species labeled with a 15-min radioactive uridine pulse just prior to extraction should show similar amounts of virus-specific RNA made in the permissive and nonpermissive hosts unless RNA degradation was occurring extremely fast (24). However, gel

[1] Present address: Department of Microbiology, College of Medicine, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pa. 17033.

analysis of 15-min pulsed RNA, at 5 h after infection, showed only low amounts of single-stranded RNA being synthesized in MDBK cells as compared to L cells (24). This paper reinvestigates this contradiction and expands our previous study to include other aspects of viral restriction.

MATERIALS AND METHODS

Cell culture. HeLa, MDBK, and L cells were routinely maintained in the laboratory. Cells were seeded onto plastic tissue culture dishes (60 by 15 mm; Falcon) in Earle minimal essential medium (MEM; GIBCO) supplemented with antibiotics, non-essential amino acids (GIBCO), and 5% fetal calf serum (GIBCO or International Scientific Ind., Inc.). Confluent monolayers (48 h) contained 2×10^6 to 4×10^6 cells per dish.

Mengovirus propagation and purification. Mengovirus was routinely propagated on L cell monolayers as previously described (23), except that the incubation period was extended to 24 h.

Virus was prepared for gel electrophoresis by harvesting lysates that had been labeled with 5 μCi of ^{14}C-labeled protein hydrolysate per ml (54 mCi/mA; Amersham/Searle) at 8.5 h after infection. Lysates were adjusted to pH 7.0 and centrifuged to remove cell debris. The clarified lysate was made 5% with respect to polyethylene glycol (Carbowax 6000), held on ice for 30 min, and then centrifuged at 15,000 $\times$ *g* for 30 min. The pellet was resuspended in 4 ml of RSB (0.01 M Tris-hydrochloride, 0.01 M NaCl, 0.0015 M $MgCl_2$, pH 7.4), and this material was loaded onto 15-ml, 5 to 45% sucrose-RSB linear gradient. Gradients were centrifuged in the SW27.1 rotor at 27,000 rpm for 3 h at 4 C in the Beckman L2-65B. Fractions were collected, and portions were analyzed for acid-insoluble radioactivity. Fractions containing presumptive virions were pooled and dialyzed for 24 h at 5 C against buffer. Dialyzed samples were then spun in the SW65 rotor at 50,000 rpm for 3.75 h at 4 C. Pellets were resuspended and stored at −20 C until used.

Plaque assay. Mengovirus was assayed on L cell monolayers overlaid with 0.7% Noble agar (Difco) in Earle MEM supplemented with 2% fetal calf serum. After 48 h of incubation at 37 C monolayers were stained with 1% crystal violet in 20% ethanol, and plaques were counted.

Hemagglutination. Mengovirus was also assayed by hemagglutination of human type O erythrocytes. Freshly drawn blood was mixed with 3.8% sodium citrate in a ratio of 9:1, washed three times with sterile 0.85% saline, and diluted to 2×10^7 to 5×10^7 erythrocytes per ml with borate buffer (0.12 M KCl, 0.05 M H_2BO_3 [pH 8.0] with 1 N NaOH) (6). This buffer was also used as the virus diluent.

Infectious center assay. Infectious centers were assayed by the method of Darnell and Sawyer (7).

Labeling of viral RNA. Viral RNA accumulation was monitored, in the presence of 5 μg of actinomycin D per ml, by labeling mengovirus-infected cells with [^{3}H]uridine (New England Nuclear) at 1 h after infection. Cells were labeled in the presence of 0.2 mM uridine (specific activity of 10 μCi/mmol) in Earle MEM containing 5% dialyzed calf serum. Viral RNA was sampled by disrupting the cell monolayer with RSB containing 0.2% sodium dodecyl sulfate (SDS) and precipitating 1×10^6 cell equivalent with cold 10% trichloroacetic acid. For sucrose gradients and gel electrophoresis, cell monolayers were labeled at ½ h after infection with 1 ml of Earle MEM containing 4 μCi of [^{3}H]uridine (specific activity of 20 μCi/mmol). Three hours later cells were removed from the glass surface with a rubber policeman, collected on ice, and washed with Hanks balanced salt solution (HBSS). The cell pellet was resuspended with 10 ml of acetate buffer (0.01 M acetate plus 0.15 M NaCl, pH 5.1) containing 1 mg of bentonite per ml and made 0.2 with respect to SDS. An equal volume of acetate buffer-saturated phenol was added, and the suspension was incubated at 37 C with agitation for 5 min. After centrifugation the aqueous phase was removed, extracted again with acetate buffer-saturated phenol, precipitated with 95% ethanol, and stored at −20 C.

Sedimentation analysis of phenol-extracted viral RNA. Mengovirus RNA samples prepared as above were diluted into 0.5 ml of SDS buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride, 0.001 M EDTA, and 0.5% SDS, pH 7.4). These samples were then applied to 11-ml linear gradients of 15 to 30% sucrose in SDS buffer and spun (285,000 $\times$ *g*) at 24 C for 5.25 h. Gradients were fractionated, and 100-μl portions were analyzed for acid-insoluble radioactivity.

Amino acid incorporation. The rate of protein synthesis was determined by 15-min pulses with 1 μCi of ^{14}C-labeled protein hydrolysate per ml in Earle MEM lacking amino acids.

Polysome labeling and isolation. Viral polysomes were analyzed by the method of Gielkens et al. (9). Mengovirus-infected monolayers were pulsed for 10 min with 15 μCi of ^{14}C-labeled protein hydrolysate per dish (60 by 15 mm). Pulses were terminated by the addition of 50 μg of cycloheximide (Sigma) per ml followed by a 10-min incubation at 37 C. Monolayers were rinsed twice with HBSS and trypsinized, and the cells collected on ice, washed twice with HBSS, and then resuspended in 0.3 ml TKM (0.05 M Tris-hydrochloride, 0.15 M KCl, and 0.007 M magnesium acetate, pH 8.5). A 0.3-ml portion of 1% Nonidet-P40 (Shell Chemical Co.) in TKM was added to the sample, which was then made 0.025% in DOC and held on ice for about 5 min with constant agitation. The lysed cells were then centrifuged at 8,000 rpm for 20 min in the Sorvall RC-2B, and the supernatant was retained.

A 0.5-ml pad of 50% sucrose in TKM and a 10.5-ml linear gradient of 26–36% sucrose in TKM were loaded with the cell lysate supernatant and centrifuged at 285,000 $\times$ *g* for 70 min at 4 C. Fractions were collected from the bottom and analyzed for acid-insoluble radioactivity.

Subviral particle analysis. A modification of the method of Phillips (15) was used to examine the subviral particles that may be involved in virion assembly. At 3.25 h after infection, 2×10^7 to 4×10^7 mengovirus-infected cells were labeled with 17 μCi of ^{14}C-labeled protein hydrolysate in the absence of cold

amino acids. Cells were incubated at 37 C a further 3.5 h, at which time radioactive medium was decanted, and cells were removed with a rubber policeman, collected, and washed twice with HBSS. Cells were resuspended in 0.9 ml RSB, allowed to swell for 20 min on ice, and ruptured with 25 strokes in a tight-fitting glass dounce homogenizer. Samples were frozen in a CO_2-ethanol bath and stored overnight at -70 C.

Samples were thawed at 37 C and spun at 2,000 rpm, and the supernatant was made 0.5% with respect to SDS. Samples were layered onto a 15-ml 5 to 45% sucrose linear gradient and centrifuged and fractionated as described.

Polyacrylamide gel electrophoresis. Polyacrylamide-urea-SDS gels (10 cm) and samples were prepared by the procedure of Raff et al. (19) and Raff and Kaumeyer (20). Gels were subject to electrophoresis for 4 h at 3.5 mA/gel, frozen, sliced into 1-mm sections, and placed in scintillation vials. To each vial 0.2 ml of 30% H_2O_2 was added, and vials were capped and incubated overnight at 60 C. Scintisol (Isolab, Inc.)-toluene (2 ml; 1:1) and 10 ml of scintillation fluid were added to count for radioactivity.

Analysis of acid-insoluble material. Samples from experiments monitoring the rate of synthesis, the accumulation of viral RNA, and the rate of amino acid incorporation were precipitated with cold 10% trichloroacetic acid with the addition of 0.05 ml of 0.5% bovine albumin as carrier. These samples were washed twice with cold 5% trichloroacetic acid, and the pellets were resuspended in 0.5 ml of Nuclear Chicago solvent and 10 ml of 2,5-diphenyloxazole-toluene scintillation fluid (6 g of 2,5-diphenyloxazole per liter of toluene). Samples from gradient fractions were precipitated as above. However, these samples were collected onto 0.44-μm HAWP membrane filters (Millipore Corp.). Radioactivity was monitored in an LS-150 Beckman scintillation counter.

RESULTS

Because mengovirus-infected HeLa cells were used as the permissive host in this study, rather than L cells (4, 23, 24), several control experiments were run to confirm that mengo infection of HeLa cells was similar to that in L cells. Single-step growth experiments (data not shown) of mengovirus in HeLa and MDBK cell monolayers showed an eclipse period of 4 h, followed by a 3-h period of exponential virus synthesis, with all virus being synthesized by 8 h after infection. HeLa cells produced 1,300 to 1,500 mengovirus progeny per cell, whereas MDBK cells produced 2 to 4 mengovirus progeny per cell. Measurement of viral-induced cytopathology by the ability of mengovirus-infected HeLa and MDBK cells to exclude the vital dye trypan blue showed that both the permissive and nonpermissive hosts died at the same rate—90% dead after 15 h.

Additional experiments confirmed (23, 24) that mengovirus infection of restrictive MDBK cells does proceed in a normal fashion for the first several hours of the virus replicative cycle. Measurement of the rate of mengovirus irreversible eclipse in MDBK cells by the method of Holland (10) suggests that mengovirus uncoating is occurring normally, as only 1% of the initial mengovirus infectivity is recoverable after a 170-min incubation period at 37 C. Another indication that a picornavirus infection is progressing normally is the inhibition of host RNA and protein synthesis, which occurs prior to the time when levels of virus-specific RNAs and proteins become detectable (11).

Mengovirus-specific RNA accumulation. Mengovirus-specific RNA accumulation was compared in MDBK cells and HeLa cells by labeling with 4 μCi of [^{3}H]uridine per ml at ½ h after infection in the presence of 5 μg of actinomycin D per ml. Cell equivalents (1×10^6) were analyzed for total acid-insoluble label, for ribonuclease-resistant label, and for heat-resistant, ribonuclease-resistant label (Fig. 1A to D) as detailed by Plagemann (17).

In this experiment 1,500 PFU were produced in each HeLa cell, whereas MDBK cells produced 4 PFU/cell, about a 400-fold difference in infectious mengovirus production. However, the RNA accumulation data show only about a 10-fold difference in the amount of total viral RNA (Fig. 1A) and virion RNA (Fig. 1D) made in the productive and restrictive hosts. The 40-fold discrepancy in the amount of virion RNA and in the amount of infectious virus, as determined by plaque assay on L cell monolayers, was not readily understandable. However, since picornaviruses produce a large ratio of physical particles for every infectious unit observed (21), the virus yields were also assayed for total particles by hemagglutination of human type O erythrocytes. It was found (Table 1) that mengovirus from MDBK cells have 400 physical particles for every infectious virion, whereas mengovirus from HeLa cells have only 10 physical particles for every infectious particle. This 40-fold increase in physical particles per infectious virion in mengovirus-infected MDBK cells reduces the differences in relative virus particle yield to 10-fold when compared with HeLa cells, in agreement with the relative amounts of uridine accumulated into virion RNA (Fig. 1D).

Using the particle to PFU ratio, as calculated in Table 1, and the data in Fig. 1, the efficiency

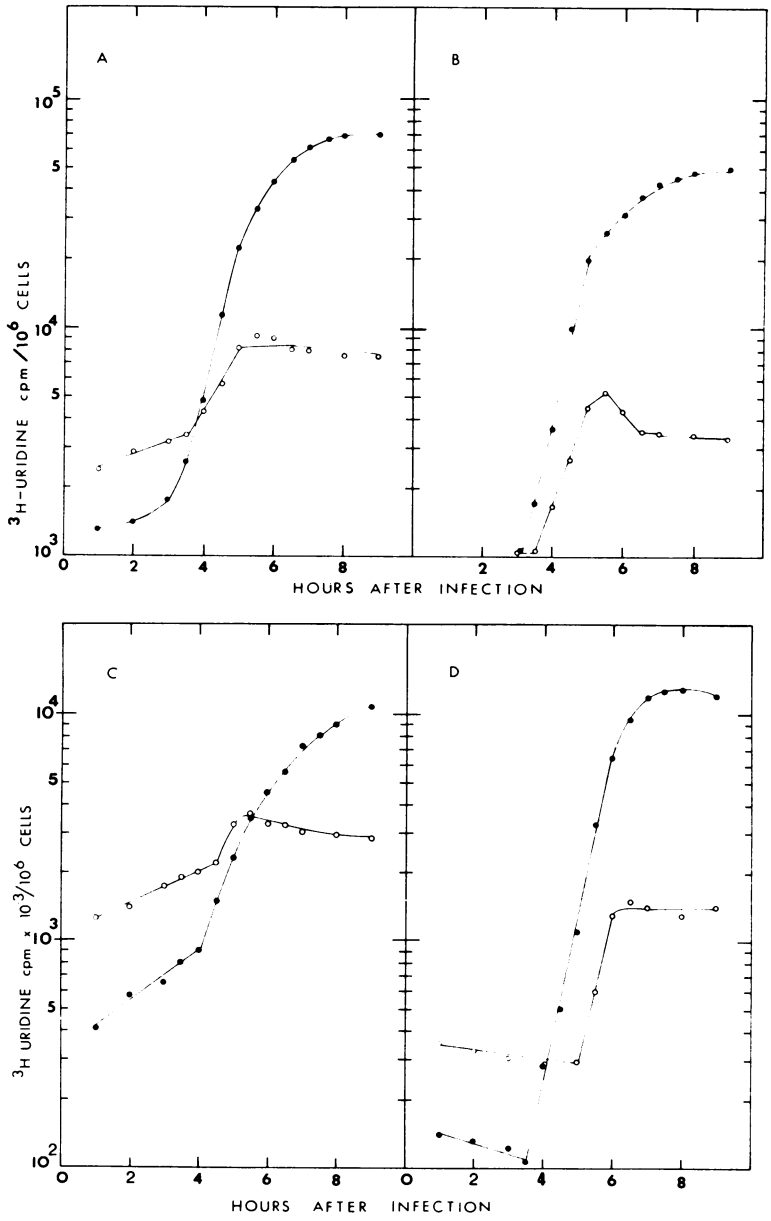

FIG. 1. *Accumulation of [^{3}H]uridine into mengovirus RNA species as measured by the method of Plagemann (17). MDBK cells and HeLa cells, infected with 100 mengovirus/cell in the presence of 5 μg of actinomycin D per ml, were labeled with 4 μCi of [^{3}H]uridine (specific activity of 20 μCi/mmol) per ml at ½ h after infection. Samples were analyzed for total viral RNA (1A), viral single-stranded RNA (1B), viral double-stranded RNA (1C), and virion RNA (1D). Symbols: ●, viral RNA accumulated in HeLa cells; ○, viral RNA accumulated in MDBK cells.*

of the permissive and restrictive hosts can be compared in terms of the relative numbers of molecules of the various RNA species synthesized (Table 2). These calculations are based on the assumption that all RNA molecules are uniformly labeled with [^{3}H]uridine. Mengovirus-infected HeLa cells accumulate 10-fold more total RNA (Fig. 1A) and virion RNA (Fig. 1D) than do mengovirus-infected MDBK cells. If there is a numerical relationship between the

TABLE 1. *Calculation of the particle-PFU ratio of mengovirus grown in MDBK and HeLa cells by hemagglutination of human type O erythrocytes and plaque assay on L cell monolayers*

Cell type	Input PFU (plaque assay)	Hemagglutination end point	No. erythrocytes/well	No. particles (calculation)	Particles:PFU
MDBK	3×10^4	4	3×10^6	1.2×10^7	1.2×10^7/ 3.0×10^4 (400:1)
HeLa	1.25×10^8	768	2×10^6	1.5×10^9	1.5×10^9/ 1.25×10^8 (12:1)
HeLa	1.25×10^8	512	2×10^6	1.024×10^9	1.024×10^9/ 1.25×10^8 (8.5:1)

TABLE 2. *Comparison of types and relative numbers of RNA molecules synthesized in mengovirus-infected HeLa and MDBK cells*

RNA determinations[a]	Mengovirus made in HeLa cells	Mengovirus made in MDBK cells	HeLa / MDBK[b]
PFU/cell	1,500	4	~400 (375)
Particles/PFU	10	400	0.025
Total virions (PFU · particles)/cell	15,000	1,600	~10 (9.48)
cpm in virion RNA[c] / virions = cpm/molecule virion ssRNA	12,000[c] / 15,000 = 0.8	1,500[c] − 300[d] / 1600 = 0.75	~1 (1.06)
cpm in total RNA[c] / cpm/molecule · ssRNA = No. ssRNA equivalents made	70,000[c] / 0.8 = 87,500	8,500[c] − 2,500[d] / 0.75 = 8,000	~10 (10.93)
cpm in RNase-resistant RNA[c] / (2) (cpm/molecule ssRNA) = No. RNase-resistant RNA molecules made	11,000[c] / (2) (0.8) = 6,875	3,300[c] − 1,200[d] / (2) (0.75) = 1,400	~5 (5.6)
cpm in ssRNA[c] / cpm/molecule ssRNA = No. ssRNA molecules made	48,000[c] / 0.8 = 60,000	4,500[c] − 900[d] / 0.75 = 4,800	12.5
total virions/cell / No. ssRNA molecules made = ssRNA packaged into virions (%)	15,000 / 60,000 = 25	1,600 / 4,800 = 33	0.767

[a] cpm, Counts per minute.
[b] Numbers in parentheses indicate exact calculation.
[c] Numbers taken from Fig. 1.
[d] Background subtracted from measurements made in MDBK cells. Numbers taken from data in Fig. 1.

number of molecules of each type of viral RNA in a cell and the final number of virions produced by that cell, then it would seem that the relative amount of single- and double-stranded RNA synthesized in MDBK cells would also be 10-fold less than that accumulated in mengovirus-infected HeLa cells. This is not the case. Rather, MDBK cells infected with mengovirus accumulates only about fivefold less double-stranded RNA than do infected HeLa cells, whereas 12.5-fold less single-stranded RNA is produced in the restrictive host (Table 2). Thus the ability to accumulate single-stranded RNA is more severely impaired.

The accumulation of [^{3}H]uridine into mengovirus RNAs in the productive and restrictive hosts is also shown in Fig. 1. Note that the accumulation of total RNA (Fig. 1A) and single-stranded RNA (Fig. 1B) in mengovirus-infected HeLa cells is exponential until 5 h after infection, at which time it becomes linear. The switch from exponential to linear synthesis of this RNA is not seen in infected MDBK cells (Fig. 1A and B); rather, at 5 h after infection total RNA and single-stranded RNA stop accumulating.

Rate of mengovirus RNA synthesis. The rate of synthesis of mengovirus RNA was compared in infected HeLa and MDBK cells in the presence of 5 μg of actinomycin D per ml, utilizing 15-min pulses with 4 μCi of [^{3}H]uridine (specific activity of 4 mCi/mmol) per ml. The data (Fig. 2) are in good agreement with the viral RNA accumulation data (Fig. 1). Although the initial rate of RNA synthesis in mengovirus-infected MDBK cells is about the same as that in infected HeLa cells, this rate declines at 4.5 h after infection. The rate of RNA synthesis in virus-infected HeLa cells is constantly increasing until 7 h, at which time it also begins to decline.

Rate of amino acid incorporation. The rate of amino acid incorporation in mengovirus-infected HeLa and MDBK cells was measured utilizing 15-min pulses with 1 μCi of ^{14}C-labeled protein hydrolysate per ml. The data (Fig. 3) show a rapid decrease in the initial rate of amino acid incorporation, representing the virus-mediated inhibition of host protein synthesis (11), and, in the case of mengovirus-infected HeLa cells, a burst of amino acid incorporation beginning at 4 h and becoming maximal at 5.5 h after infection, which represents mengo-specific protein synthesis. This burst of amino acid incorporation is absent in mengovirus-infected MDBK cells.

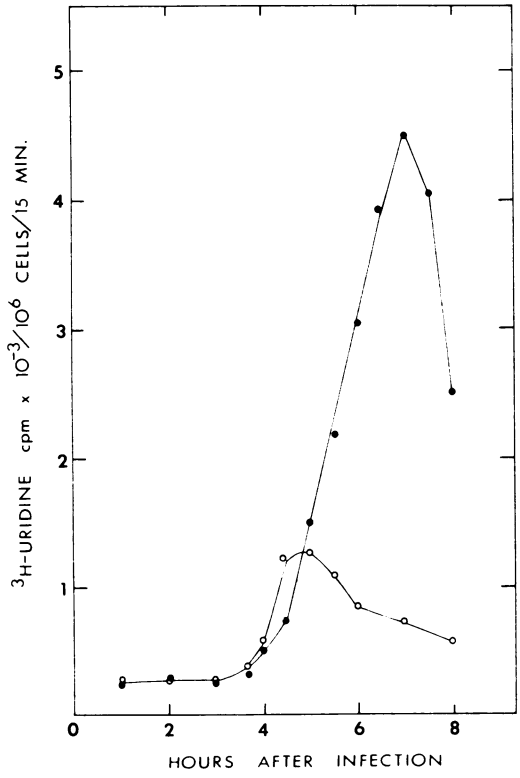

FIG. 2. *Rate of mengovirus-specific RNA synthesis. MDBK and HeLa cells, infected with 100 PFU of mengovirus/cell in the presence of 5 μg of actinomycin D per ml, were pulsed for 15 min, at the indicated times, with 4 μCi of [^{3}H]uridine per ml. Samples were analyzed for uridine incorporation into acid-insoluble material. Symbols: ●, mengovirus RNA in HeLa cells; ○, mengovirus RNA in MDBK cells.*

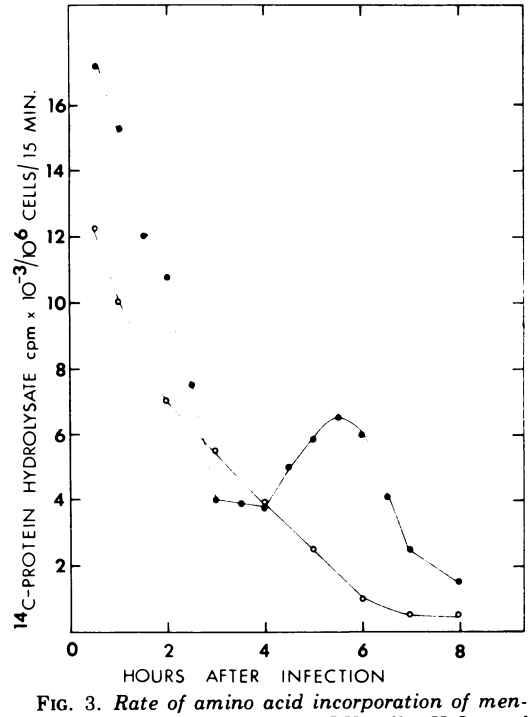

FIG. 3. *Rate of amino acid incorporation of mengovirus-infected HeLa and MDBK cells. HeLa and MDBK cells, infected with 180 PFU of mengovirus/cell in the presence of 5 μg of actinomycin D per ml, were pulsed for 15 min with 1 μCi of ^{14}C-labeled protein hydrolysate per ml at the indicated times. Samples were analyzed for hot, acid-insoluble amino acids. Symbols: ●, amino acid incorporation in mengovirus-infected HeLa cells; ○, amino acid incorporation in mengovirus-infected MDBK cells.*

Virus-specific polysomes. Mengovirus-specific polysomes were examined in HeLa and MDBK to answer the question of whether viral translation is occurring in the restricted host at later times in the infectious cycle and to ascertain the relative amount of viral protein being synthesized in MDBK compared to the permissive host. Polysomes were resolved in a linear 26 to 36% TKM sucrose gradients (9) after a 10-min pulse with 15 μCi of ^{14}C-labeled protein hydrolysate per ml. Control experiments with polysomes from mengovirus-infected HeLa cells indicated that the kinetics of polysome labeling followed the pattern observed in Fig. 3. Polysome profiles from mengovirus-infected HeLa and MDBK, pulsed for 10 min with radioactive amino acids at 5 h after infection, are illustrated in Fig. 4. There is a shift in polysome profile from host polysomes (fractions 12 to 18) to viral-induced polysomes (fractions 5 to 9), with a sedimentation coefficient of 350 to 400S in both the restrictive and permissive host. Whether the differences noted in the two cell lines between fractions 10 to 20 is significant awaits further experimentation. These polysomes extracted from HeLa cells appear to be about threefold more active in de novo protein synthesis than those extracted from MDBK cells if one assumes that the level of amino acid labeling reflect rate of protein synthesis. If it is assumed that the rates of ribosome initiation and protein chain elongation are the same in both the permissive and restrictive hosts, then the relative amount of radioactive amino acid incorporation into viral polysomes can be considered a measure of the relative amount of functional mengovirus mRNA present in the respective polysomes.

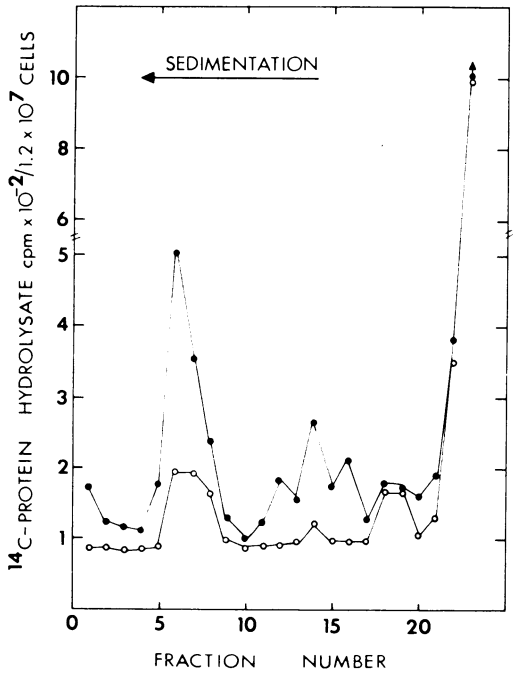

FIG. 4. *Sedimentation analysis of polysomes from mengovirus-infected HeLa and MDBK cells. Mengovirus-infected HeLa and MDBK cells, in the presence of actinomycin D, were pulsed with 15 μCi of ^{14}C-labeled protein hydrolysate for 10 min at 5 h after infection. Pulses were terminated and polysomes were extracted as described in Materials and Methods. Cell lysates were loaded onto 11-ml linear gradients of 26 to 36% sucrose in TKM and centrifuged in the SW41 rotor at 41,000 rpm (285,000 × g) for 70 min at 4 C in the Beckman L5-65. Fractions were analyzed for acid-insoluble amino acids. Symbols: ●, mengovirus-infected HeLa cells; ○, mengovirus-infected MDBK cells.*

Virus maturation. If single-stranded viral RNA synthesized in mengovirus-infected MDBK cells is restricted by 12.5-fold (Fig. 1B, Table 2) over that observed in the permissive host, resulting in a 10-fold reduction in virions (Fig. 1D) and a 3-fold reduction in functional message (Fig. 4), it might be predicted that an examination of the particles involved in virus maturation would reveal a large accumulation of previral particles in the restricted host. Mengovirus-infected HeLa and MDBK cells were labeled with 3.4 μCi of ^{14}C-labeled protein hydrolysate from 3.25 to 6.75 h after infection in the presence of actinomycin D. Cells were prepared for sedimentation analysis by a modification of the method of Phillips (15) as described above, and the profiles obtained are illustrated in Fig. 5.

Based on the reported sedimentation coefficient of 160S for mengovirus (13), sedimentation values of 125S and 80S were estimated for the slower sedimentation particles detected on the gradients. To insure that the 160S particle was indeed mengovirus, large quantities of ^{14}C-labeled amino acid-labeled virus were isolated by the same gradient procedure, further concentrated as described, and subject to electrophoresis on 10% polyacrylamide-SDS-urea gels at 3.5 mA/gel for 4 h. The gel profile (Fig. 6) shows three peptides, equally labeled with amino acids, which correspond to the alpha, beta, and gamma peptides, reported to be the major proteins of the mengovirus capsid (25). A minor capsid peptide, epsilon, was also detected, but the other minor peptide of the mengovirus capsid, delta, with a reported mol wt of 7,000 to 10,000 (25), was presumably electrophoresed off the gel. Whereas the 125S particle was present in approximately equal amounts in the re-

stricted and nonrestricted hosts, the 160S particle was dramatically reduced and the 80S particle dramatically increased in the MDBK cells.

DISCUSSION

Inefficient picornavirus replication has been attributed to abortive early interactions of the virus with potential host cells (7, 10). A second type of inefficient picornavirus replication occurs when the virus is able to initiate its replicative cycle, but some characteristic of the host limits the final amount of progeny virus manufactured (4, 22–24). The restricted multiplication of mengovirus in MDBK cells is an example of this latter type of nonpermissive picornavirus replication.

Previous experiments have indicated that mengovirus infection occurs in all MDBK cells of a culture and that all the early events in the viral replicative cycle are occurring.

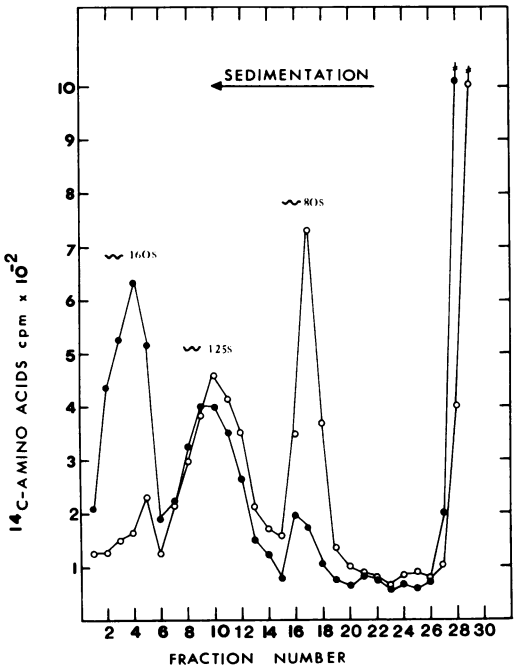

FIG. 5. *Sedimentation analysis of virions and subvirion particles from mengovirus-infected HeLa and MDBK cells. Infected cells were labeled with 3.4 μCi of ^{14}C-labeled protein hydrolysate per ml from 3.5 to 6.75 h after infection in the presence of actinomycin D. Cell lysates were prepared and sedimented through linear 5 to 45% sucrose in RSB gradients in the SW27.1 rotor at 27,000 rpm for 3 h at 4 C. Gradients were fractionated from the bottom, and fractions were analyzed for isotope in acid-insoluble material. Sedimentation coefficients were estimated, assuming intact mengovirus to be 160S (13). Symbols: ●, mengovirus-infected HeLa cells; ○, mengovirus-infected MDBK cells.*

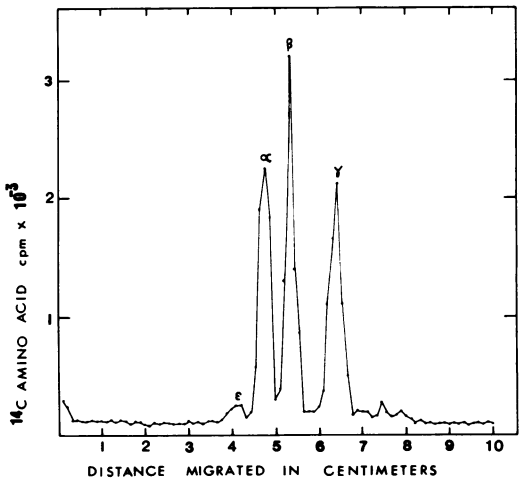

FIG. 6. *Polyacrylamide-SDS-urea gel electrophoresis (10%) of purified ^{14}C-labeled amino acid-labeled mengovirus. Electrophoresis was for 4 h at 3.5 mA/gel.*

Restricted MDBK cells produce 2 to 7 infectious mengovirus per cell, whereas the permissive host, HeLa cells, synthesize 1,300 to 1,500 infectious mengovirus per cell, about a 400-fold difference between the two hosts. This discrepancy in virus yield is deceptively large and does not agree with data showing only a 10-fold difference in the amount of virion RNA (Fig. 1D) and in the amount of virion particles (Fig. 5). The hemagglutination test, used to measure the number of physical virus particles synthesized in each host, coupled with the plaque assay of infectious mengovirus (Table 2), indicate that MDBK cells make 400 physical particles for each infectious virus produced, whereas HeLa cells produce about 10 physical particles for each infectious particle. This 40-fold increase in physical particles per infectious mengovirus in MDBK cells reduces the difference in relative virus particle yield to 10-fold, agreeing with the virion RNA data (Fig. 1D) and the protein particle data (Fig. 5). The significance of this large excess of physical particles is not clear.

As has been previously reported, at 4 to 5 h postinfection in MDBK cells there is a cessation in the accumulation of viral RNA (23, 24). In HeLa cells, 5 to 8 h after infection is the time when the largest amount of single-stranded RNA is synthesized, approximately 60,000 molecules, of which 30% is packaged into about 90% of the virions. Less than 10% of the virions in HeLa cells are manufactured prior to 5 h, and these contain about 5% of the single-stranded

RNA accumulated prior to 5 h. No RNA is packaged into virions in MDBK cells prior to 5 h, and no new single-stranded RNA is synthesized after 5 h. All the RNA packaged into capsid protein in mengovirus-infected MDBK cells after 5 h is from the small amount of RNA synthesized prior to 5 h, 33% of which is packaged. Mengovirus-infected MDBK cells appear to lack the late phase of the RNA biosynthetic process that contributes over 90% of the single-stranded RNA to virions in the productive host. Although the final virion yield in MDBK cells is 10-fold less than the yield in mengovirus-infected HeLa cells, the number of virions assembled in both hosts that contain RNA synthesized prior to 5 h is almost the same.

RNA synthesis in picornavirus-infected cells is known to be associated with two smooth membrane complexes (1, 5). The smaller RNA replicating complex, sedimenting at 70*S*, is composed of RNA that is about 70% resistant to ribonuclease digestion (1, 5). About 60% of the RNA in this lighter complex is complementary RNA (5). The heavier RNA replicating complex is 100 to 300*S* and contains predominantly 35*S* viral RNA (1, 5), with only about 8% of the RNA in this complex being complementary RNA (5). These observations suggest that two functionally distinct RNA replicative complexes are operative during the picornavirus replicative cycle. It is not known whether the function of these two complexes are coordinated, whether the polymerases involved in the RNA biosyntheses are the same or whether both complexes are equally functional throughout the virus replicative cycle. Interpretation of the mengovirus restriction data in terms of the two RNA replication complexes would suggest that the lighter replication complex, synthesizing predominantly double-stranded RNA, is functional in MDBK cells prior to 5 h. However, the heavier complex, which may be primarily responsible for the large amount of single-stranded viral RNA synthesized after 5 h, as seen in mengovirus-infected HeLa cells, may not be functional at all in mengovirus-infected MDBK cells.

Maturation particles larger than 14*S* and smaller than 160*S* have never been reported for any of the cardioviruses. However, Fernandez-Tomas and Baltimore (8) have reported a 125*S* particle in poliovirus-infected HeLa cells, the provirion, which contains the 35*S* viral RNA genome and a full complement of proteins that have not yet undergone their final cleavage. The poliovirus empty capsid, with the same protein composition as the provirion, but without the viral RNA genome, is a particle with a 73*S* sedimentation coefficient (16). Recently Miller and Plagemann (12) have identified an 80*S* ribonucleoprotein particle that is a degradation product of mengovirus which has been subjected to heavy UV irradiation. This ribonucleoprotein contains mengo RNA and about 30% of the capsid proteins alpha, beta, and gamma.

It is not known if the 125*S* and 80*S* particles observed in Fig. 5 contain the mengovirus genome, nor is the polypeptide composition of these particles known. A 125*S* particle found in BEV-infected L cells does contain viral RNA (R. T. Su and M. W. Taylor, manuscript in preparation). However, the relative distribution of these particles extracted from mengovirus-infected HeLa and MDBK cells do support other observations reported in this communication. Virions (160*S*) from mengovirus-infected HeLa cells do accumulate in a 10-fold excess over those made in MDBK cells. Particles (80*S*) from MDBK cells have accumulated in a five-fold excess over 80*S* particles from the permissive host. This could be due to either a defect in the maturation process or result from a limiting supply of viral single-stranded RNA. Whether these are two independent events remains to be explored. Interestingly, the intermediate-sized particle sedimenting at 125*S* is present in about equal amounts in both the permissive and restrictive hosts at a time (6.75 h) when almost all mengovirus that will be made in MDBK cells has already been synthesized (Fig. 1D).

Whether this 125*S* peak is responsible for the hemagglutination activity and whether it is a defective viral particle or provirion requires further investigation.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant CA10417 from the National Cancer Institute. S.O.P. is a predoctoral trainee supported by Public Health Service Microbiology training grant GM503 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Arlinghaus, R. B., J. J. Syrewicz, and W. T. Loesch. 1972. RNA polymerase complexes from mengovirus infected cells. Arch. Gesamte Virusforsch. **38**:17-28.
2. Baltimore, D. 1969. The replication of picornaviruses, p. 101-176. *In* H. B. Levy (ed.), Biochemistry of viruses. Marcel Dekker, New York.
3. Baltimore, D., M. Girard, and J. E. Darnell. 1966. Aspects of the synthesis of poliovirus RNA and the formation of virus particles. Virology **29**:179-189.
4. 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.
5. Caliguiri, L. A. 1974. Analysis of RNA associated with the poliovirus RNA replication complexes. Virology **58**:526-535.

6. Craighead, J. E., and A. Shelokov. 1961. Encephalomyocarditis virus hemagglutination-inhibition test using antigens prepared in HeLa cell cultures. Proc. Soc. Exp. Biol. Med. **108**:823-826.
7. Darnell, J. E., and T. K. Sawyer. 1960. The basis for variation in susceptibility to poliovirus in HeLa cells. Virology **11**:665-675.
8. Fernandez-Tomas, C. B., and D. Baltimore. 1973. Morphogenesis of poliovirus. II. Demonstration of a new intermediate, the provirion. J. Virol. **12**:1122-1130.
9. Gielkens, A. L. J., T. J. M. Berns, and H. Bloemendal. 1971. An efficient procedure for the isolation of polyribosomes from tissue culture. Eur. J. Biochem. **22**:227-231.
10. Holland, J. J. 1962. Irreversible eclipse of poliovirus by HeLa cells. Virology **16**:163-176.
11. McCormick, W., and S. Penman. 1967. Inhibition of RNA synthesis in HeLa and L cells by mengovirus. Virology **31**:135-141.
12. Miller, R. L., and P. G. W. Plagemann. 1974. Effect of ultraviolet light on mengovirus: formation of uracil dimers, instability and degradation of capsid, and covalent linkage of protein to viral RNA. J. Virol. **13**:729-739.
13. Newman, J. F. E., D. J. Rowlands, and F. Brown. 1973. A physicochemical sub-grouping of the mammalian picornaviruses. J. Gen. Virol. **18**:171-180.
14. Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell. 1963. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger-RNA. Proc. Natl. Acad. Sci. U.S.A. **49**:654-662.
15. Phillips, B. A. 1969. *In vitro* assembly of poliovirus. I. Kinetics of the assembly of empty capsids and the role of extracts from infected cells. Virology **39**:811-821.
16. Phillips, B. A. 1971. *In vitro* assembly of poliovirus. II. Evidence for the self-assembly of 14s particles into empty capsids. Virology **44**:307-316.
17. Plagemann, P. G. W. 1968. Reversible inhibition of induction of mengovirus RNA polymerase and of virus maturation in Novikoff rat hepatoma cells by phenethyl alcohol. Virology **34**:319-330.
18. Plagemann, P. G. W., and H. E. Swim. 1966. Replication of mengovirus. I. Effect of synthesis of macromolecules by host cell. J. Bacteriol. **91**:2317-2326.
19. Raff, R. A., G. Greenhouse, K. W. Gross, and P. R. Gross. 1971. Synthesis and storage of microtubule proteins by sea urchin embryos. J. Cell Biol. **50**:516-527.
20. Raff, R. A., and J. F. Kaumeyer. 1973. Soluble microtubule proteins of the sea urchin embryo: partial characterization of the proteins and behavior of the pool in early development. Dev. Biol. **32**:309-320.
21. Schwerdt, C. E., and J. Fogh. 1957. The ratio of physical particles per infectious unit observed for poliomyelitis viruses. Virology **4**:41-52.
22. Sturman, L. S., and I. Tamm. 1969. Formation of viral ribonucleic acid and virus in cells that are permissive or nonpermissive for murine encephalomyelitis virus (GDVII). J. Virol. **3**:8-16.
23. Wall, R., and M. W. Taylor. 1969. Host-dependent restriction of mengovirus replication. J. Virol. **4**:681-687.
24. Wall, R., and M. W. Taylor. 1970. Mengovirus RNA synthesis in productive and restrictive cell lines. Virology **42**:78-86.
25. Ziola, B. R., and D. G. Scraba. 1974. Structure of the mengo virion. I. Polypeptide and ribonucleate components of the virus particle. Virology **57**:531-542.