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Host-Dependent Restriction of Mengovirus Replication[1]

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Mengovirus infection of a restrictive cell line, Maden's bovine kidney (MDBK), results in a virus yield 1,000-fold less than that obtained from productively infected cell lines such as L cells or Ehrlich ascites tumor cells (EAT). Cells of both types of host systems are infected with comparable efficiencies and are completely killed as a consequence of infection. Infective center assays, coupled with the observation of total cell killing, suggest that comparable numbers of cells synthesize viral antigen and release virus in both types of host system. Viral-specific ribonucleic acid (RNA) synthesis is initiated and proceeds in an identical fashion for approximately 4 hr after the infection of MDBK, EAT, or L-cells. At this time, viral RNA synthesis in MDBK ceases, whereas viral RNA synthesis in EAT and L-cells continues at a linear rate. These results indicate that none of the early viral events leading to the initiation of viral-specific RNA synthesis constitutes the primary site of mengovirus restriction in MDBK. Rather it appears that the cessation of viral RNA synthesis in restrictive cells constitutes the primary limiting event. Based on its delayed interaction with mengovirus RNA synthesis, it appears that the host-related restrictive agent is initially compartmentalized and then released as a consequence of infection subsequent to those early events in mengovirus infection leading to the initiation and continued synthesis of viral RNA.

Buck et al. (6) reported that mengovirus and Bovine enterovirus-1 were capable of initiating infection in a wide variety of cultured animal cells. Despite the appearance of comparable cytopathic effects in all the infected cell lines, the yield of progeny virus varied over 1,000-fold, depending on the host system employed. Estimates, made late in the replication cycle, of the amounts of viral antigen and infectious ribonucleic acid (RNA), indicated that both were produced in amounts approximately proportional to the ultimate virus yield of a given host system. Holland (10) subsequently reported that both the relative proportions and the relative rates of synthesis of picornavirus proteins were not influenced by the host system. Only the total amount of viral protein synthesized reflected the capacity of various cell systems to replicate picornaviruses. These results would suggest that the host-related intracellular barriers to picornavirus replication limit to the same extent the expression of all the information of the input viral genome. In addition, the finding (6) that the pattern of host cell susceptibility to mengovirus differs from the pattern of host cells productively infected by Bovine enterovirus-1 suggests that the host-related intracellular barriers to picornavirus replication are quite specific. Since those cellular factors limiting, or stimulating, the replication of certain picornaviruses may also be control agents in normal cellular metabolism, the determination of the basis of picornavirus restriction may result in some understanding of the control of differential gene expression in normal cells.

To establish the nature of the restrictive event, we began a systematic comparison of the replicative events of mengovirus in restrictive and productive host systems. In this paper, we present evidence which indicates that the interruption of viral RNA synthesis, subsequent to its initiation and early rise, constitutes the primary site of mengovirus restriction in Maden's bovine kidney (MDBK) cells.

MATERIALS AND METHODS

Cell culture. Cells were grown in Eagle's Minimum Essential Medium (MEM) containing 5% calf serum and supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and mycostatin (5 units/

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ml). Mouse L-cells were kindly supplied by Clayton Buck, and MDBK was purchased from Flow Laboratories (Rockville, Md.).

Ehrlich ascites tumor cells (EAT) were maintained by intraperitoneal passage of about 10^8 cells at 8-day intervals in adult Swiss white mice or by freezing at −70 C in 10% glycerol solution in MEM.

Virus infection and assays. Infections were carried out in cell suspensions at concentrations of 10^7 cells/ml in MEM without serum, containing actinomycin D at 5 μg/ml. In all experiments, cells were infected with an added multiplicity of infection (MOI) of about 20 plaque-forming units (PFU)/cell. Viral adsorption was allowed to proceed for 45 min to 1 hr at 37 C under conditions of low-speed rotary shaking. Cells were washed three times in MEM with 5% calf serum and were then suspended in the same medium containing 5 μg of actinomycin D per ml at a cell concentration of 10^6 to 2×10^6 cells/ml. Infected cultures were maintained on a rotary shaker at about 150 rev/min.

L-cell monolayers for mengovirus plaque assays were prepared by seeding 2-oz screw-cap bottles with about 5×10^6 cells and incubating them at 37 C overnight. After infection, assay monolayers were overlaid with washed agar (Difco) at a final concentration of 0.6% in MEM with 5% calf serum. Plaques from virus titrations were counted after 48 to 60 hr by fixing and staining the monolayers with a solution of 1% crystal violet in 20% ethyl alcohol by the method of Buck et al. (6). Determinations of infectious centers was performed by the method of Brownstein and Graham (5). Washed, infected cells were allowed to attach to L-cell monolayers for 2 hr prior to overlayering with 0.6% washed agar in MEM with 5% calf serum. Infectious-center assays were terminated and stained, as described in the plaque assay, some 48 to 72 hr after infection. Mengovirus hemagglutination assays were carried out at room temperature by the addition of 2×10^6 freshly drawn human type O red blood cells in 0.05 ml of 0.15 M NaCl to successive 1:2 serial dilutions of viral preparations in 0.05 ml of 0.15 M NaCl. The ratio of PFU to hemadsorption unit was found to be 10^5 to 2×10^5. It was further found that this ratio did not change throughout the course of the mengovirus replication cycle, thereby suggesting that infectivity and hemagglutin activity are identical entities.

Kinetics of cell killing. Viable cell counts were carried out by mixing cells with trypan blue solution and estimating the number of stained cells by direct count in a hemocytometer (14).

Kinetics of viral-specific RNA synthesis. Cells were infected at 10^7 cells/ml in the presence of 5 μg of actinomycin D per ml. At 1 hr postinfection, cells were diluted to about 2×10^6 cells/ml with MEM containing 5% calf serum and 5 μg of actinomycin D per ml. ^{3}H-uridine at 1 μc/ml (20 to 30 c/mmole; New England Nuclear Corp., Boston, Mass.) was added at this time. At various times after the addition of ^{3}H-uridine, duplicate 0.5 ml-samples were pipetted into 2.0 ml of 10% trichloroacetic acid and precipitated 1 hr at 0 C. Precipitates were washed twice with 5% trichloroacetic acid, suspended in 0.5 ml of Solvent (Nuclear-Chicago Corp., Des Plaines, Ill.), and solubilized overnight at 37 C. Radioactivity was counted in a scintillation counter (Beckman Instruments Co., Fullerton, Calif.) after suspension of the solubilized samples in toluene-2,5-diphenyloxazole-1,4-bis-2-(5-phenyloxazolyl)-benzene (toluene-PPO-POPOP) scintillation fluid.

Pulse-labeling and agarose-acrylamide gel electrophoresis of viral RNA. Infected cells (10^8) were pulse labeled with 100 μc of ^{3}H-uridine (20 to 30 c/mmole, New England Nuclear) for 30 min either at 2.5 or 5 hr after infection. Uptake of radioactive uridine was halted by pouring the cells onto an equal volume of frozen 0.15 M NaCl and then washing the cells three times with cold 0.15 M NaCl. Washed cells were resuspended to about 4×10^7 cells/ml in reticulocyte standard buffer [0.01 M tris(hydroxymethyl)amino methane (Tris) hydrochloride (*p*H 7.5), 0.01 M NaCl, 0.0015 M $MgCl_2$], and a Dounce cytoplasmic extract was prepared by the method of Penman, Becker, and Darnell (13). The postnuclear fraction was centrifuged at 20,000 × *g* for 30 min for the isolation of the viral replicative complex (2). The pellet resulting from this centrifugation was suspended in 2.0 ml of E buffer [0.02 M sodium acetate, 0.04 M Tris, 0.001 M ethylenediaminetetraacetate, made to *p*H 7.2 with glacial acetic acid (4)] containing 0.2% sodium dodecyl sulfate (SDS). Viral RNA was released from the resuspended complex by single phenol-SDS extraction at 37 C. The aqueous phase was precipitated overnight with ethyl alcohol and centrifuged, and the pellet was dissolved in 0.1 ml of E buffer containing 20% sucrose and 0.2% SDS. Samples prepared in this fashion were frozen at −70 C until application to gels for electrophoresis.

Agarose (0.5%)-acrylamide (2.0%) gels were prepared by the method of Peacock and Dingman (12) modified by substituting E buffer and incorporating 0.2% SDS in the gels. Gels were allowed to polymerize in glass tubes (0.7 by 12 cm) bearing a tight-fitting Teflon plug in one end. After polymerization of the gels, the open ends of the tubes were covered with a piece of dialysis tubing secured with a rubber band. Removal of the Teflon plug served to loosen the gels from the walls of the tubing and facilitated removal of the gels upon completion of the run.

Gels were prerun for 1 hr at a constant 10 ma/gel in E buffer plus 0.2% SDS. Samples of viral RNA were loaded in volumes of 10 to 30 μliters and electrophoresis was carried out at a constant 10 ma/gel for 3 hr at room temperature. At this time, gels were removed from the tubes and sliced into 2-mm slices by means of a razor-blade device. Viral RNA was released from gel slices for counting of radioactivity by separating the slices into scintillation vials containing 0.5 ml of a solution composed of BioSolv-3 (Beckman Instruments Co.) diluted 1:2 with toluene-PPO-POPOP scintillation fluid. Sealed vials were then incubated at 37 C for 12 to 15 hr. Radioactivity was counted after the addition of 5 ml of the toluene-PPO-POPOP scintillation fluid.

RESULTS

Low yields of mengovirus from infected MDBK could result either from limited virus production

in all the cells in an infected culture or from the production of high titers of virus in a few sensitive cells in an otherwise resistant cell population. To distinguish between these two possibilities, we compared the efficiency of infectious-center formation in L-cells and MDBK infected with mengovirus. The results (Table 1) indicate that comparable numbers of cells in mengovirus-infected cultures of both MDBK and L-cells produce and release virus. The efficiency-of-plating values reported in Table 1 for both host systems are consistent with previously reported values for mengovirus-infected L-cells which ranged from 0.35 to 0.80 (5). The low efficiency-of-plating values probably reflect the inefficient attachment of infected cells to the assay monolayers. Since the infection of both cell lines was carried out under standard conditions, these data also suggest that both L-cells and MDBK are infected with essentially identical efficiencies.

Buck et al. (6) reported that comparable cytopathic effects, as assessed by the destruction of cell monolayers, were observed in all infected cell lines regardless of their capacity for virus production. We examined the kinetics of cell lysis in a more quantitative fashion by employing trypan blue viable staining (Fig. 1). In L-cells and MDBK, the initiation of cell disintegration occurs at about 4 hr after infection. In another cell line, Ehrlich ascites tumor (EAT), viral disruption of the cell membrane with concomittant cell staining does not begin until around 8 hr after mengovirus infection. However, in all three host systems, essentially all cells are killed by 24 hr after the addition of mengovirus.

Although the identity of the viral protein resulting in cell killing is not known, it is known that the synthesis of viral antigen is required for cell killing (1). Thus, the quantitative cell killing noted in all three cell lines, coupled with the comparable efficiency of infectious center formation in L-cells and MDBK, strongly suggests that all the cells in a mengovirus-restrictive host system such as MDBK are infected, synthesize viral antigen, and release low levels of mengovirus.

Single-step growth experiments. The widely disparate yields of mengovirus in productive host systems such as mouse L-cells or EAT, when compared to virus production in a restrictive host system such as MDBK, are evident from the single-step multiplication cycles presented in Fig. 2. The mengovirus production in MDBK is exceeded several thousand-fold by the yields noted in L-cells and EAT. Employing an inoculum of proflavine-containing mengovirus which was inactivated subsequent to infection, Buck et al. (6) demonstrated that the low levels of virus produced in restrictive hosts did not represent residual viral inoculum. In spite of the great differences in ultimate virus yield, the kinetics of intracellular virus appearance, as well as the time of release of free virus, are essentially identical in L-cells and MDBK. In both L-cells and MDBK, the eclipse period extends to 3 to 4 hr after infection. At this time, intracellular virus production commences in both cell lines, culminating in the maximum intracellular virus titers being reached about 6 hr after infection. The release

TABLE 1. *Assay of infectious centers (IC) after mengovirus infection of MDBK and L cells*

Host system	No. of assay plates	Expected IC per plate	Range of observed IC per plate	Range of EOP[a] values
MDBK...	20	50	15–22	0.30–0.44
L cells....	25	50	16–29	0.32–0.58

[a] Efficiency of plating is defined as observed IC per expected IC.

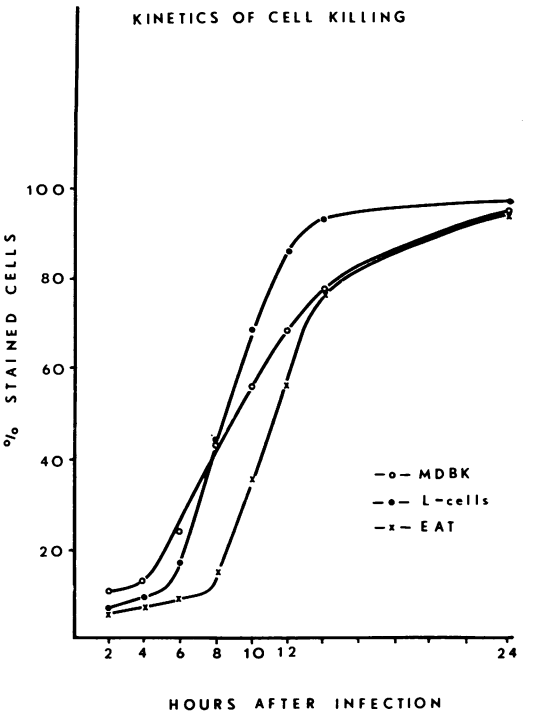

FIG. 1. *Kinetics of cell killing after mengovirus infection. Cells were infected, as noted in Materials and Methods, with an added MOI of about 20 PFU/cell. At intervals after infection, 0.5-ml samples containing about 10^6 cells were stained with trypan blue solution, and the percentage of nonviable cells was determined by direct microscopic count. Uninfected, actinomycin D-treated cells exhibit only a 10 to 20% decrease in viability during the time of viral-induced cell killing.*

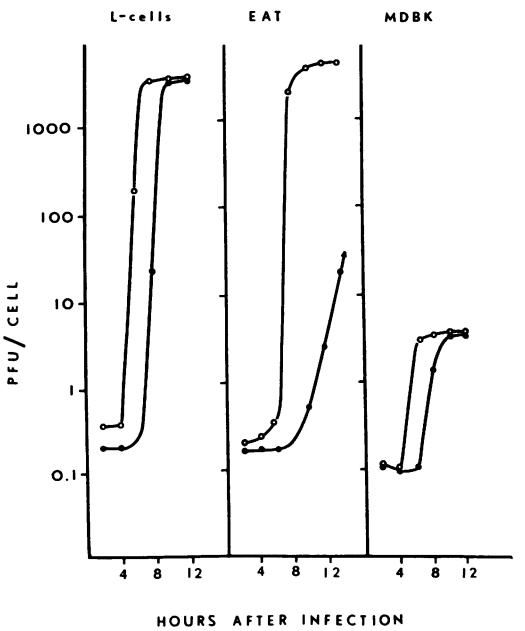

FIG. 2. *Single-step growth curves of mengovirus in L-cells, EAT, and MDBK. Mengovirus infection of cells was carried out as described in Materials and Methods. Symbols:* ○, *total virus released by two cycles of freeze-thaw;* ●, *virus free in the supernatant fluid after the infected cells were removed by centrifugation.*

of intracellular virus begins at 6 hr after infection in both MDBK and L-cells, with the maximal titers of released virus being reached 2 to 3 hr later. Whereas the eventual mengovirus yield from EAT is comparable to that obtained from L-cells, the events of virus production, accumulation, and release in this host system are delayed several hours relative to the events in L-cells. In EAT, intracellular virus first appear at 6 hr after infection, with the maximal titers of intracellular virus occuring some 2 to 3 hr later. Virus release in mengovirus-infected EAT begins at about 8 hr post infection, whereas the maximal titers of released virus appear at 16 to 20 hr after infection.

These data from the single-step growth experiments indicate that the late replicative events concerned with maturation and release of mengovirus appear to be identically functional in both L-cells and MDBK, despite the great difference in virus yields. The delay in the viral replicative events noted in EAT relative to L-cells demonstrates that even among productively infected hosts, considerable variation can exist in the expression of virus-specific functions.

Viral-specific RNA synthesis. The studies of Baltimore, Girard, and Darnell (3) indicated that the maximal rate of poliovirus RNA synthesis was the same over an MOI range of 1 to 30 PFU per cell. However, at the lower MOI, a longer time interval after infection elapsed before the maximal rate was attained. We reasoned, therefore, that if the number of functional input mengovirus templates in restrictive cells was reduced, either as a consequence of inefficient uncoating or some other early limiting event or as a consequence of early ribonuclease activity, then a delay in the initiation of maximal viral RNA synthesis should be observed. Fig. 3 shows the kinetics of viral RNA synthesis in L-cells and MDBK when cellular RNA synthesis is depressed by the addition of actinomycin D. In both L-cells and MDBK, the initiation and early exponential increase in trichloroacetic acid-precipitable counts in viral RNA is identical. This finding suggests that viral RNA synthesis is initiated with equivalent amounts of template in both systems and

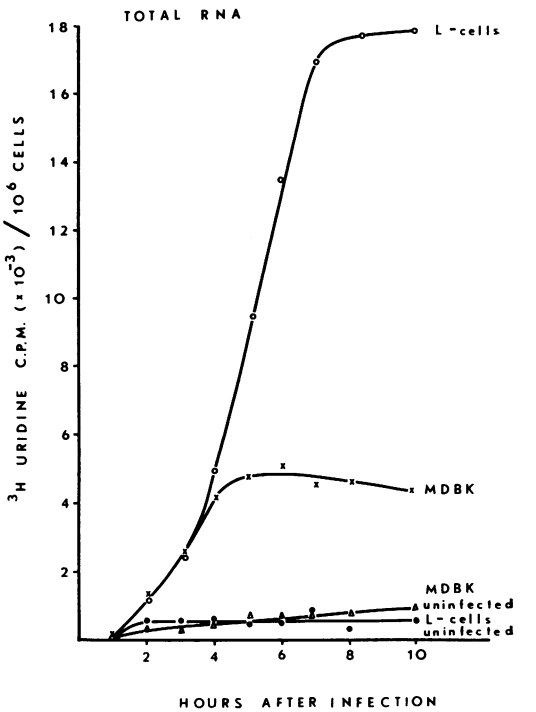

FIG. 3. *Kinetics of total viral-specific RNA synthesis. Mengovirus infection was carried out as noted in Materials and Methods. Actinomycin D at 5 μg/ml was present from the time of addition of virus. At 1 hr after infection, ^{3}H-uridine at 1 μc/ml was added. At intervals thereafter, duplicate 0.5-ml samples containing about 10^6 cells were removed and analyzed for cold trichloroacetic acid-precipitable label. Uninfected controls were otherwise treated in the same fashion as the experimental samples.*

reinforces the concept that the primary restrictive event does not occur until after uncoating of the viral RNA. However, at the time when viral RNA synthesis in L-cells enters into the phase of linear increase, viral-specific RNA synthesis in MDBK appears to cease.

The results presented in Fig. 3 indicate that the final amount of viral RNA present in MDBK corresponds to about 25% of the amount present in L-cells. Since the results presented in Fig. 2 indicate approximately a thousandfold difference in the number of infectious virus produced, it appears that a large portion of the trichloroacetic acid-precipitable counts persisting after the apparent halt in MDBK may be biologically inactive, relatively high molecular weight, viral RNA.

The apparent shut-off of viral RNA synthesis in MDBK at some 4 hr postinfection was examined by pulsing infected cells with tritiated uridine at 2.5 to 3 hr after infection and after the apparent restrictive shut-off at 5 to 5.5 hr postinfection. The results of the pulse-labeling experiments, as analyzed by agarose-acrylamide gel electrophoresis, are presented in Fig. 4. The most rapidly migrating species is the single-stranded viral RNA, followed by the replicative form or extracted viral RNA of double-stranded character. Migrating most slowly is the replicative intermediate, a double-stranded complex bearing nascent single-stranded viral RNA. A critical verification of the identity of these viral RNA species will be presented elsewhere. However, as might be expected from their proposed properties, both single-stranded RNA and replicative intermediate are eliminated by ribonuclease digestion. Label in the ribonuclease-resistant core of the replicative intermediate (presumably double stranded in character) is not degraded but appears to be converted to material migrating in the replicative form portion of the profile. The peak designated as replicative form is not degraded as a result of ribonuclease incubation (Fig. 5). In both L-cells and MDBK pulsed at 2.5 to 3 hr after infection, the three viral RNA species implicated in picornavirus replication are present in essentially identical quantities, thereby verifying the results of Fig. 3 which indicated that early

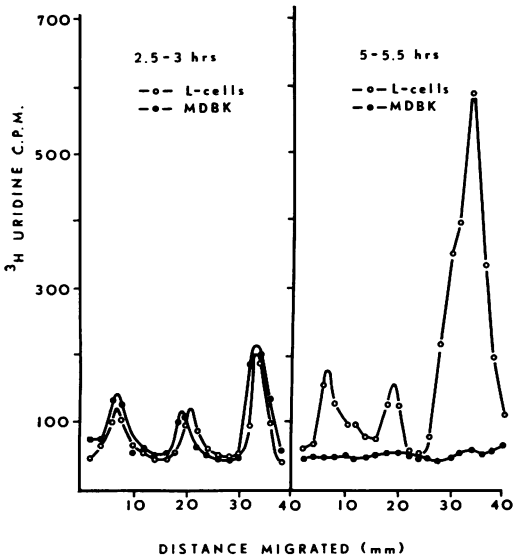

FIG. 4. *Analysis of pulse-labeled mengovirus RNA by agarose-acrylamide gel electrophoresis. Infected cells were pulsed with ^{3}H-uridine, and viral RNA was extracted as noted in Materials and Methods. Loading volume was 10 μliters, equivalent to about 10^6 infected cells. The 2.0% acrylamide-0.5% agarose gels were run at 10 ma/gel for 3 hr at room temperature. Symbols: O, L-cells; ●, MDBK.*

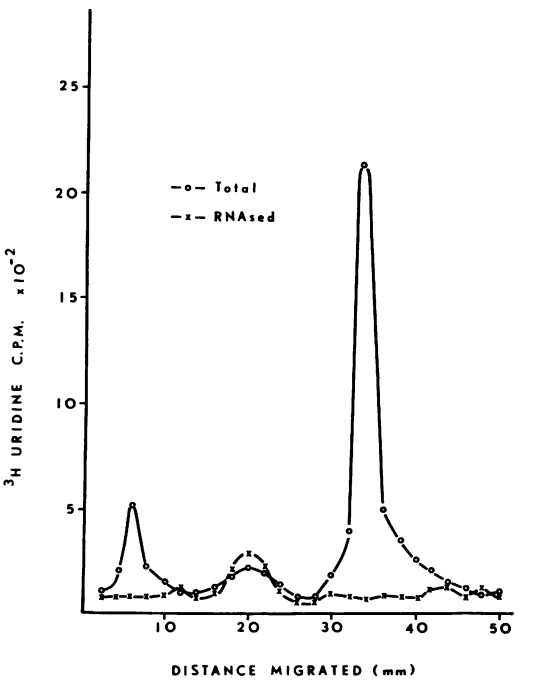

FIG. 5. *Agarose-acrylamide gel electrophoresis profile of mengovirus RNA from EAT. Infected EAT cells were labeled continuously with ^{3}H-uridine from 1 to 5.5 hr after infection. Actinomycin D at 5 μg/ml was present from the beginning of infection. Ribonuclease digestion was carried out using 10 μg ribonuclease per ml at 27 C for 30 min. Ribonuclease digestion was conducted in reticulocyte standard buffer (see Materials and Methods). SDS-phenol extracted, ethyl alcohol-precipitated viral RNA was run on 2.0% acrylamide-0.5% agarose gels at 10 ma/gel for 3 hr at room temperature. Loading volume was 20 μliters, or about 2 × 10^6 infected cell equivalents.*

viral RNA synthesis proceeded in identical fashion in both productive and restrictive cell lines. In L-cells pulsed after the time of the apparent shut-off of RNA synthesis noted in MDBK, i.e., from 5 to 5.5 hr postinfection, the three species of viral RNA are observed, though with a proportionately greater amount of label in single-stranded viral RNA. In contrast, in MDBK pulsed from 5 to 5.5 hr after infection little, if any, viral RNA synthesis is observed.

DISCUSSION

Previously examined instances of inefficient or abortive replication of enteroviruses have been attributed to restrictive events occurring early in the infectious cycle such as negligible adsorption (11, 15) or the inefficient uncoating of the viral RNA (7, 9).

The results of Buck et al. (6) which indicated that all the cells in a restrictive host for mengovirus appeared to contain low levels of viral antigen as detected by the fluorescent-antibody technique, as well as the results from our infectious center assays (Table 1) and determinations of the kinetics and extent of cell killing (Fig. 1), suggest that essentially all the cells in a restrictive population are infected, synthesize a low level of viral antigen, and release a limited number of infectious virus particles. Thus, neither inefficient infection nor the infection of only a limited number of sensitive cells in an otherwise resistant population seems to be the explanation of the restrictive replication of mengovirus in MDBK.

Should any of the viral infectious events prior to uncoating of the viral RNA prove to be the primary restrictive site, a delay in the time of initiation and possibly a reduction in the rate of expression of certain early viral-specific functions should be observed. Therefore, the identical early expression of mengovirus RNA synthesis (Fig. 3) in both restrictive and productive host systems suggests that the restrictive event in MDBK does not occur until after the initiation of viral-specific biosynthesis. The hypothesis that the involvement of the primary restrictive event occurs subsequent to the early events in infection is supported by the report of Buck et al. (6) and our own unpublished results, which indicate that the early mengovirus function responsible for the inhibition of host protein synthesis is identically expressed in both MDBK and L-cells. Additional unpublished results in our laboratory indicate that the initiation and early increase in another virus-coded activity, the viral RNA polymerase, occurs in similar fashion in both L-cells and MDBK. These results suggest that inefficient uncoating is not the explanation for mengovirus restriction in MDBK; rather, the data presented in Fig. 3 and 4 indicate that the cessation of viral RNA synthesis at about 4 hr after infection constitutes the primary restrictive event in mengovirus-infected MDBK.

The nature of the primary host-dependent restrictive component is not known. The results in Fig. 3 indicate that the total viral-specific uptake in MDBK is some 25% of that noted in L-cells. Since the data in Fig. 2 show about a thousand-fold difference in the virus yield of MDBK relative to L-cells, it follows that much of the acid-insoluble viral-specific label observed in MDBK must be biologically inactive, high molecular weight RNA. These combined results implicate the interaction of ribonuclease with the viral RNA synthesized in restrictive cells. The question of whether ribonuclease involvement constitutes the primary restrictive event or whether viral RNA synthesis is first shut-off, while ribonuclease degradation of the product represents a secondary restrictive event, is presently under investigation. In either case, based upon its delayed interaction with mengovirus RNA synthesis, it appears that the host-related restrictive agent or ribonuclease is initially compartmentalized and then released as a consequence of infection, subsequent to those early events in infection leading to the initiation and continued synthesis of viral RNA.

Cellular organelles such as the cell nucleus and lysosomes are known to be grossly altered late in the normal course of enterovirus replication. However, certain enzymatic activities normally localized in the nucleus [e.g., nicotinamide adenine dinucleotide (NAD) pyrophosphorylase] have also been shown to be released without nuclear degradation into the cytoplasm 3 to 4 hr after encephalomyocarditis virus infection (8). Such release occurs several hours in advance of degenerative changes in the nucleus detectable by electron microscopy. Traub et al. (16) reported that the apparent selective release of nuclear NAD pyrophosphorylase into the cytoplasm appeared to be temporally related to the rise in encephalomyocarditis viral RNA polymerase activity in the cytoplasm. Such data suggest a possible involvement of host nuclear components in forming or maintaining an active viral polymerase complex.

Activation and release of lysosomal enzymatic activities in virus-infected cells occurs concomittant with the appearance of virus-induced cytopathic effects (17). The results in Fig. 1 indicate that the onset of cell killing, presumably as a consequence of extensive lysosomal breakdown, occurs in a similar manner in mengovirus-infected L-cells and MDBK. However, it is conceivable that mengovirus infection of MDBK might trigger the early selective release of lysosomal ribonucleases or other lysosomal activities. That the mengovirus-induced labilization of MDBK

lysosomes or cell nuclei, resulting in the selective release of specifically localized ribonucleases or other components involved in the regulation of cellular RNA synthesis, might constitute the restrictive event is currently under investigation.

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