Foot-and-Mouth Disease Virus-Induced Alterations of Baby Hamster Kidney Cell Macromolecular Biosynthesis: Inhibition of Ribonucleic Acid Methylation and Stimulation of Ribonucleic Acid Synthesis

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The kinetics of ribonucleic acid (RNA) and protein synthesis and RNA methylation were examined after foot-and-mouth disease virus (FMDV) infection of baby hamster kidney cells. The synthesis of RNA extracted from the whole cells was stimulated two- to threefold above the control level of synthesis. This increased rate was attributed to viral RNA synthesis. The inhibition of host RNA methylation was concomitant with more pronounced than protein synthesis inhibition. The methylation of transfer RNA was initially inhibited by virus infection, but rose to within 70 to 80% of the control level just prior to the production of maximal amounts of virus-specific RNA polymerase. Cycloheximide studies showed that rapid cessation of protein synthesis did not result in the immediate cessation of RNA methylation. A comparison between the kinetics of inhibition of these processes by cycloheximide and FMDV infection suggests that FMDV selectively inhibits RNA methylation.

MATERIALS AND METHODS

Chemicals. \(^{3}H\)-labeled (methyl) methionine (specific activity, 76 mc/mnmole) was obtained from New England Nuclear Corp., Boston, Mass.; \(^{14}C\)-uridine (specific activity, 30 mc/mnmole) was obtained from Schwarz BioResearch Inc., Orangeburg, N.Y.; and guanidine hydrochloride and cycloheximide were obtained from Sigma Chemical Co., St. Louis, Mo.

Cell growth and virus infection. BHK cells, strain 21, clone 13 (American Type Culture Collection, Rockville, Md.), were grown in 2-liter Baxter bottles (6 X 10\(^{6}\) to 8 X 10\(^{6}\) cells per bottle) by the method of Polatnick and Bachrach (12).

BHK cells were infected with purified FMDV (type A, strain 119) 5 to 6 days after passage by using a virus-cell multiplicity of 20 as previously described (2). Growth medium (12), on both noninfected and infected cells, was replaced with a complete tissue culture medium containing essential amino acids, vitamins, 0.2% glucose, 0.03% glutamine, and 10% dialyzed bovine serum. Isotopic pulse labeling of cells was carried out by adding 1 mc of \(^{14}C\)-uridine or 30 mc of \(^{3}H\)-(methyl) methionine, or both, to each culture 15 or 30 min before harvesting. The isotopic pulse medium contained one-tenth the normal level of unlabeled methionine to preclude any methionine deprivation effects (16). Guanidine hydrochloride

458
was used to inhibit the synthesis of viral RNA without interfering with virus-induced host protein synthesis inhibition (11, 13). Cycloheximide was used to inhibit BHK cell protein synthesis (15). When used, guanidine hydrochloride (0.002 M) or cycloheximide (0.002 M) was added at zero time to the growth medium.

Cells were harvested by cooling the bottles to 0°C in an ice bath, suspending the cells in 50 ml of a solution containing 0.10 M NaCl, 0.05 M KCl, 0.005 M MgCl₂, 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5), and 0.5% bovine serum albumin, and centrifuging at 1,600 × g.

Extraction procedure and isotopic specific activity measurements. RNA was extracted from whole cells and from nuclear and cytoplasmic subfractions. Briefly, these fractions were obtained by suspending the cell pellet in 10 volumes of hypotonic buffer (0.05 M NaCl, 0.0036 M CaCl₂, 0.02 M Tris, pH 7.5) for 3 min at 0°C, homogenizing with a loose-fitting Dounce homogenizer, and subsequently making the suspension hypertonic [0.25 M sucrose, 0.1 M KCl, 0.005 M MgCl₂, 0.02 M Tris (pH 7.5), 0.5% Lubrol WX]. The nuclear pellet obtained by centrifugation at 1,600 × g was washed twice with 10 volumes of hypotonic buffer containing 0.05% sodium deoxycholate and 0.5% Lubrol WX. All supernatant liquids (cytoplasmic) were combined and saved for subsequent phenol extraction. The nuclear pellet was extracted at 55°C with detergent-phenol, and the RNA-containing aqueous-phase material was treated with deoxyribonuclease as described elsewhere (3). Protein was precipitated from the phenol phase with 4 volumes of cold methanol-ether (1:1, v/v), washed twice with methanol-ether, suspended in 10 volumes of 1 N NaOH (14), and reprecipitated with 5% trichloroacetic acid.

Solubilization of the methanol-ether-precipitated pellet with 1 N NaOH caused no significant loss of [H]-(methyl) methionine label upon reprecipitation with 5% trichloroacetic acid, whereas the 14C-uridine label remained solubilized. The rates of RNA synthesis and methylation were measured by obtaining the 14C-uridine and H-methyl label incorporated into RNA. As determined by alkaline hydrolysis and ribonuclease sensitivity, this fraction had little or no 1H-(methyl) methionine incorporated into protein.

Radioactivity measurements were made on protein and RNA samples precipitated with 5% trichloroacetic acid and washed onto membrane filters (Schleicher & Schuell Co., Keene, N.H.). The samples were dissolved in 0.5 ml of 1 N NaOH and counted in a model 4322 Tri-Carb spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) as previously described (2). Counting efficiencies for 14C in the presence of H were 48 and 9%, respectively.

Protein concentrations were determined by the biuret reaction. RNA concentration was determined by its adsorption at 260 nm using a 1% extinction coefficient of 240. Adsorption measurements were made with a Gilford model 2000 spectrophotometer.

Trichloroacetic acid-insoluble, isotopic-specific activities of the RNA and protein fractions extracted from FMDV-infected and noninfected BHK cells are presented as per cent of the noninfected control: (isotopic specific activity infected/isotopic specific activity control) × 100.

RESULTS

Protein synthesis. The kinetics of total cellular protein synthesis after FMDV infection are given in Table 1. At 210 min postinfection (PI), the point of maximal viral RNA polymerase production (11), protein synthesis was inhibited 52% whereas at the time of maximal virus release [300 min PI (13)] it was inhibited 83%. Similar kinetics were observed for cytoplasmic protein synthesis (see Fig. 2). More extensive studies of FMDV inhibition of acidic and basic nuclear protein fractions (Vande Woude, unpublished data) agreed (±5%) with the kinetics shown in Table 1. Thus, the synthesis of both the nuclear and cytoplasmic proteins is inhibited at a similar rate and to about the same extent throughout the infectious cycle.

RNA synthesis. The kinetics of total cellular RNA synthesis, determined by the incorporation of 14C-uridine into RNA (Table 1), show stimulation after FMDV infection. This stimulated synthesis (254% at 210 min PI) is associated with the cytoplasmic fraction (Fig. 2), the site of FMDV replication (1), and FMDV replicase enzyme production (11). Evidence presented in Table 1. Kinetics of total cellular protein synthesis, RNA synthesis, and RNA methylation in FMDV-infected BHK cells

<table>
<thead>
<tr>
<th>Time postinfection</th>
<th>Protein synthesis</th>
<th>RNA synthesis</th>
<th>RNA methylation</th>
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<tr>
<td>min</td>
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<td>90</td>
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<td>240</td>
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<tr>
<td>300</td>
<td>17</td>
<td>120</td>
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a Time of isolation PI, 6 × 10⁶ control and infected cells pulse-labeled 15 min before harvest with 30 μc of H-(methyl) methionine and 1 μc of 14C-uridine.

b Average specific activity of control cell; protein synthesis equal to 4,585 counts/min of H-(methyl) methionine/mg of protein.

c Average specific activity of control cell; RNA synthesis equal to 3,626 counts/min of 14C-uridine/mg of RNA.
d Average specific activity of control cell; RNA methylation equal to 7,045 counts/min of H-(methyl)/mg of RNA.

e Guanidine (0.002 M) added at zero time.
Fig. 1. Sucrose gradient profiles of total cellular RNA extracted from noninfected and FMDV-infected BHK cells labeled for 15 min with $^3$H-(methyl) methionine and $^{14}$C-uridine. About 1 mg of RNA was layered on a 5 to 25% linear sucrose gradient, centrifuged for 17 hr at 63,500 × g in an SW25.1 rotor, and analyzed as previously described (14). Figures 1A and 1C are noninfected control cell RNA profiles extracted after incubation for 180 and 300 min, respectively. Figures 1B and 1D are profiles of RNA extracted from FMDV-infected cells at 180 and 300 min PI, respectively. The direction of sedimentation is from right to left.

Fig. 1 and Table 2 shows that the stimulated incorporation of $^{14}$C-uridine into RNA can be attributed to the synthesis of virus-specific RNA.

**RNA methylation.** Total cellular RNA methylation, like protein synthesis, is rapidly inhibited in the infected cell (Table 1). However, there is a consistently greater decrease in RNA methylation than protein synthesis. Thus, at 180 min RNA methylation is inhibited 65%, whereas protein synthesis is inhibited 43%. The relationships between these two processes may be more than fortuitous since it has been shown that FMDV inhibition of ribosomal precursor RNA methylation rapidly interferes with the appearance of new ribosomes (3).

**Effect of guanidine.** Production of an active viral RNA polymerase in BHK cells was inhibited by guanidine (11). The effect of this compound on FMDV control of total cellular RNA methylation and RNA synthesis was examined at 240 min PI (Table 1). As previously reported (13), guanidine did not alter FMDV inhibition of host protein synthesis (66% of control at 240 min PI). In contrast, RNA synthesis was no longer stimulated in the infected cell in the presence of guanidine. This is additional evidence that the stimulated level of RNA synthesis is due to virus-specific RNA synthesis. The data also show that guanidine did not alter FMDV-induced RNA methylation inhibition. Thus, at 240 min PI total cellular RNA methylation is 27% of the control in the absence of guanidine and 29% of the control in its presence.

**Sucrose gradient analysis of whole-cell RNA.** Sucrose gradient analysis of $^3$H-methyl- and $^{14}$C-uridine-labeled total cellular RNA extracted at 180 and 300 min PI after pulse labeling for 15 min shows more clearly virus-associated RNA methylation inhibition and virus-specific RNA synthesis stimulation (Fig. 1). The methyl label incorporated into 18 to 45S RNA after a 15-min pulse was shown to be rapidly inhibited after FMDV infection (3). At 180 min PI, 18 to 45S RNA methylation was only 10% of the control (Fig. 1). On the other hand, almost 50% of the total cell RNA methyl label in the infected cell was associated with the 4S region of the gradient. As will be shown (Fig. 2, 3), cytoplasmic 4S RNA methylation did not decrease continuously

![Fig. 1](image1.png)

![Fig. 2](image2.png)
TABLE 2. Half-life of inhibition of BHK RNA methylation, RNA synthesis, and protein synthesis in the presence of FMDV or cycloheximide

<table>
<thead>
<tr>
<th>Determination</th>
<th>FMDV</th>
<th>Cycloheximide(^a)</th>
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<tbody>
<tr>
<td>RNA methylation</td>
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</table>
| Nuclear ribosomal precursor RNA (45 and 32S) | 60\(^b\) | 15
| Cytoplasmic ribosomal RNA (18 and 28S) | 90\(^b\) | 8
| Cytoplasmic transfer RNA (4S) | 115-200\(^c\) | 80 |
| Nuclear RNA synthesis (45 and 32S) | \(\geq 300\(^d\) | 8
| Protein synthesis | 180-200 | 3

\(^a\) Noninfected cells incubated with (+) 0.002 M cycloheximide.
\(^b\) See reference 3.
\(^c\) Transfer RNA methylation in the FMDV-infected cell is stimulated to 72 to 80% of the control after 150 min PI.
\(^d\) FMDV infection in the presence of 0.002 M guanidine-hydrochloride.

after infection, but was stimulated to 70 to 80% of the control level at 120 to 150 min PI.

Although not shown, treating the sucrose gradient fractions with ribonuclease (1) rendered all the methyl label from both the noninfected and virus-infected cells trichloroacetic acid-soluble. This would indicate that the 20S FMDV-specific (ribonuclease-resistant) RNA (1) was not methylated during a 15-min pulse. The stimulated incorporation of \(^14\)C-uridine into total cellular RNA is associated, in part, with 37S RNA (Fig. 1). This RNA, identified as single-stranded FMDV-specific RNA (1), was stimulated more than twofold above the control level after infection. Note that there was no comparable discrete incorporation of uridine in the 37S region of noninfected cell RNA.

Kinetics of cytoplasmic RNA methylation. The data of previous reports (3, 14), as well as that in Fig. 1, indicate that viral-induced tRNA methylation inhibition did not decrease as rapidly or to the same extent as ribosomal precursor RNA methylation. To study this further, the kinetics of cytoplasmic protein synthesis, RNA synthesis, and RNA methylation were determined after infection. Noninfected and FMDV-infected cells were pulse-labeled for 15 min with \(^14\)C-uridine and \(^3\)H-(methyl) methionine before harvest at the indicated times. Since little or no \(^3\)H-methyl label was found to be associated with FMDV-specific RNA, the primary methyl-labeled cytoplasmic RNA component should be 4S RNA (5). The cytoplasmic kinetics clearly show (Fig. 2) that at 60 min PI cytoplasmic RNA methylation was only 50% of the control, whereas at 120 min PI the methyl label incorporated rose to within 72% of the control. By 240 min PI, the amount of methyl label in the cytoplasm decreased to 25%. In contrast to the stimulation of cytoplasmic RNA methylation, cytoplasmic protein synthesis continuously declined. The synthesis of RNA in the cytoplasm, the site of FMDV replication (1), was 10-fold above the control level at 240 and 300 min PI.

The higher percentage of inhibition of total cellular RNA methylation at 180 and 300 min (65 and 93%, respectively; Table 1, Fig. 1) in comparison to the cytoplasmic RNA methylation inhibition at these times (50 and 80%, respectively; Fig. 2) possibly reflects differences in the rates of RNA methylation inhibition in the nucleus and cytoplasm. It has been shown, for example, that the methylation of all nuclear RNA species is drastically inhibited after FMDV infection (3).

The period of recovery of cytoplasmic RNA methylation at 120 min PI (Fig. 2) prompted further examination of this RNA methylation. Noninfected and FMDV-infected cells were pulse labeled for 30 min with \(^3\)H-(methyl) methionine before harvest at 60, 150, and 240 min PI. It is evident from the gradient profiles in Fig. 3 that early in infection there was an apparent
inhibition of cytoplasmic 4S RNA methylation; at 150 min PI, the amount of RNA methylation in a 30-min pulse increased to 80% of the control. By 240 min PI, the amount of methyl label decreased to 20% of the control. It is also evident from these profiles that a discrete peak of methyl-labeled RNA in the 5 to 7S (labeled 7S) region of the gradient of the control was markedly inhibited by infection. The methylation of this species of RNA was not stimulated at 150 min PI as was 4S RNA.

**Effect of cycloheximide.** Cycloheximide rapidly inhibits protein synthesis at the translation level (5). Although FMDV alteration of host protein synthesis may not act at the same level, it is important to establish whether protein synthesis inhibition would effect a concomitant loss of host RNA methylation. BHK cells were incubated at zero time with and without cycloheximide (0.002 m) and were pulse-labeled for 15 min with \(^{3}H\)-(methyl) methionine and \(^{14}C\)-uridine before harvesting at intervals of 15 min for up to 2 hr. The cells were then subfractionated into nuclei and cytoplasm prior to RNA extraction. The incorporation of \(^{14}C\)-uridine and \(^{3}H\)-(methyl) label into RNA was determined on sucrose gradient-fractionated material. The incorporation of \(^{3}H\)-(methyl) methionine into protein was used to determine the rate of protein synthesis. The data are presented as the time when synthesis of RNA or protein and RNA methylation are 50% of the control level or the “half-life” \(T_{0.5}\) (Table 2). For comparison, the \(T_{0.5}\) for similar fractions is presented after FMDV infection. The data show that cycloheximide inhibited protein synthesis most rapidly, followed by nuclear RNA synthesis (2 to 3 times longer) and then by ribosomal precursor RNA methylation (5 times longer), and finally by 4S RNA methylation (13 times longer). After FMDV infection, the most rapidly inhibited process was ribosomal and ribosomal precursor RNA methylation. This was followed by the inhibition of both protein synthesis and 4S RNA methylation. The \(T_{0.5}\) for nuclear RNA synthesis, however, was greater than 300 min PI. Thus, although RNA methylation was inhibited very early in infection, RNA synthesis still continued. This order of inhibition was unlike that seen with cycloheximide (in which ribosomal precursor RNA synthesis diminished more rapidly than RNA methylation). The \(T_{0.5}\) inhibition of the methylation of tRNA by cycloheximide was the slowest of the metabolic processes studied \((T_{0.5} = 40\) min). This inhibition, however, could not be compared to FMDV infection in which there was a stimulation of tRNA methylation.

**DISCUSSION**

The stimulated incorporation of uridine into total cellular RNA, two- to threefold above the host cell control level, is in contrast to our earlier findings of RNA synthesis inhibition of 40 to 50% at 180 to 300 min PI (13). This stimulated RNA synthesis occurred concomitantly with a change to a low-pass BHK cell line. The differences in host cell response to FMDV infection may be similar to the host-dependent restrictions on mengovirus replication (R. Wall and M. W. Taylor, Bacteriol. Proc., p. 166, 1969). In a restrictive host, the yields of mengovirus and virus-specific polymerase were depressed. Thus, the high-pass BHK cell line used for our earlier studies may have been a restrictive host.

We have reported the pronounced inhibition of host RNA methylation and protein synthesis occurring late in the infectious cycle (14). The kinetic data in Table 1 show that FMDV infection causes a greater inhibition of total cellular RNA methylation than protein synthesis at any given time PI. These values were not corrected for the amount of protein synthesis associated with the viral genome. However, at 90 min PI this value is small since viral-specific polyribosomes are barely detectable (Ascione, unpublished data). By comparison, RNA methylation is reduced to 57% of the control level at this time, whereas protein synthesis is 87% of the control.

The sucrose gradient analysis (Fig. 1), together with the guanidine study (Table 1), indicated that the stimulated RNA synthesis was due to the synthesis of virus-specific RNA. By selectively inhibiting virus-specific RNA synthesis with guanidine (11), the level of inhibition of host RNA synthesis can be estimated (30% at 240 min PI). The same value was obtained for RNA extracted from the nuclei of FMDV-infected BHK cells (3). In comparison to the low level of RNA synthesis inhibition, RNA methylation and protein synthesis are inhibited 71 and 66%, respectively, at 240 min PI. This finding suggests that host-cell RNA synthesis inhibition is not responsible for the more rapidly inhibited RNA methylation and protein synthesis.

Guanidine inhibits virus-specific RNA synthesis without detectably affecting FMDV-associated protein synthesis inhibition (11, 13). Virus-induced RNA methylation inhibition, like protein synthesis inhibition, was also unaffected by guanidine. Cycloheximide rapidly inhibited protein synthesis but did not immediately affect RNA methylation. Therefore, if the cessation of RNA methylation after FMDV infection is due to protein synthesis inhibition, then it would be expected to exceed methylation inhibition in
rate and extent. Protein synthesis was not inhibited at a greater rate or extent at any time after FMDV infection. Thus, protein synthesis inhibition may be due to FMDV-induced inhibition of host RNA methylation.

The sucrose gradient profiles of whole-cell RNA (Fig. 1) revealed that the only significant level of total cellular RNA methyl label present in the FMDV-infected cell at 180 min is found in the 4S region and not in the ribosomal region. Previously, we reported that the methylation of 45S ribosomal precursor RNA was inhibited 50% by 60 min PI. Although not stressed, sucrose gradient profiles of nuclear RNA showed a profound inhibition of slow sedimenting (<10S) rapidly methylated RNA (3). This information, together with the kinetic data (Table 1, Fig. 2), indicated that the substantial level of methyl label found in the first 2 hr of infection is predominantly in the cytoplasmic 4S fraction. In contrast to the rapid and uninterrupted inhibition of nuclear RNA methylation, cytoplasmic 4S RNA methylation is first inhibited and then stimulated to within 70 to 80% of the control level at 120 to 150 min PI, and then declines (Fig. 3). The stimulated methylation of 4S RNA could be associated with the production of virus-specific proteins and enzymes, since it occurs just prior to the production of maximal levels of viral RNA polymerase (210 min PI). Hence, FMDV may be modifying host cell tRNA, which is active in the production of virus-specific proteins. This possibility is currently under investigation by the use of gel electrophoretic methods.

LITERATURE CITED


