Synthesis of Plus- and Minus-Strand RNA in Rotavirus-Infected Cells

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The genomes of the rotaviruses consist of 11 segments of double-stranded RNA. During RNA replication, the viral plus-strand RNA serves as the template for minus-strand RNA synthesis. To characterize the kinetics of RNA replication, the synthesis and steady-state levels of viral plus- and minus-strand RNA and double-stranded RNA in simian rotavirus SA11-infected MA104 cells were analyzed by electrophoresis on 1.75% agarose gels containing 6 M urea (pH 3.0). Synthesis of viral plus-strand and minus-strand RNAs was detected initially at 3 h postinfection. The steady-state levels of plus- and minus-strand RNAs increased from this time until 9 to 12 h postinfection, at which time the levels were maximal. Pulse-labeling of infected cells with [³H]uridine showed that the ratio of plus- to minus-strand RNA synthesis changed during infection and that the maximal level of minus-strand RNA synthesis occurred several hours prior to the peak of plus-strand RNA synthesis. No direct correlation was found between the levels of plus-strand and minus-strand RNA synthesis in the infected cell. Pulse-labeling studies indicated that both newly synthesized and preexisting plus-strand RNA can act as templates for minus-strand RNA synthesis throughout infection. Studies also showed that less than 1 h was required between the synthesis of minus-strand RNA in vivo and its release from the cell within virions.

Rotaviruses, members of the family *Reoviridae*, are an important cause of gastroenteritis in several species of animals (10, 12). The genomes of the rotaviruses consist of 11 segments of double-stranded RNA (dsRNA) ranging in molecular weight from 0.4×10^6 to 2.0×10^6 (9). Like viral mRNA, the plus-strand RNA in each segment is capped at the 5' terminus but lacks a 3' poly(A) sequence (13, 18). Previous studies have shown that the nucleotide sequences at the 5' and 3' termini of the 11 genome segments are conserved (13, 18). Similar to synthesis of reovirus dsRNAs (1, 22), synthesis of rotavirus dsRNAs is an asymmetrical process whereby viral plus-strand RNAs act as templates for the synthesis of minus-strand RNAs to produce dsRNAs (19).

Rotaviruses consist of two icosahedral layers (shells) of protein (21). Associated with the inner shell is an RNA polymerase which can be induced in vitro to synthesize viral plus-strand mRNAs (3, 15). The inner shell consists of a core of dsRNA and the viral proteins VP1 (125 kilodaltons [kDa]) and VP2 (41 kDa) surrounded by the major inner-shell protein VP6 (94 kDa) (2, 5). The outer shell contains the trypsin-sensitive protein VP3 (88 kDa) and the glycoprotein VP7 (38 kDa) (6, 8, 14). Rotavirus-infected cells contain several viral nonstructural proteins of unknown function (5, 17).

To provide further information on the replication of the rotaviruses, we have examined the synthesis of plus-strand RNAs (transcription) and the synthesis of minus-strand RNAs (RNA replication) in cells infected with simian rotavirus SA11 by using an electrophoretic system that allows strand separation (20). Our data indicate that the level of RNA replication in SA11-infected cells does not correlate directly with the level of viral transcription. In contrast to

the reoviruses (1), SA11 plus-strand RNA that is made at any time postinfection can act as a template for the synthesis of minus-strand RNA.

MATERIALS AND METHODS

Cell culture and virus infection. Fetal rhesus monkey kidney cells (MA104) were maintained in Eagle minimal essential medium containing 5% fetal bovine serum and 5% newborn bovine serum (19). MA104 cells were infected with plaque-purified simian rotavirus SA11 as described previously (19). SA11 was activated prior to infection by incubation for 30 min at 37°C with 5 µg of trypsin per ml (1:250; Difco Laboratories).

Preparation of ³H-labeled total RNA. Confluent monolayers of MA104 cells were infected with 5 to 10 PFU of rotavirus SA11 per cell. Upon infection, cells were maintained in serum-free minimal essential medium containing 5 µg of actinomycin D per ml to inhibit host transcription. To examine the steady-state levels of viral RNA in vivo, infected cells were continuously labeled with 50 µCi of [³H]uridine per ml (40 Ci/mmol; ICN Pharmaceuticals Inc.), beginning immediately after infection (1 h postinfection [p.i.]). Viral RNAs were pulse-labeled by the addition to cells of 50 µCi of [³H]uridine per ml (40 Ci/mmol), beginning at times indicated.

Total RNA was prepared from cells as follows. Infected cell monolayers were washed twice and then scraped into hypotonic buffer (0.01 M NaCl, 0.01 M Tris hydrochloride [pH 8.1], 1.5 mM MgCl₂). The cells were incubated for 10 min on ice and disrupted with 14 strokes of a Dounce homogenizer. Nuclei and large cellular debris were removed from the lysate by centrifugation at $12,000 \times g$ for 10 min at 4°C. The supernatant (cytoplasmic fraction) was recovered and deproteinized by phenol extraction. The total RNA was collected by ethanol precipitation and then suspended in water (45 µl). A portion (5 µl) of total-RNA samples was

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FIG. 1. Synthesis and steady-state levels of viral RNAs in rotavirus-infected cells. Total RNA was prepared from cytoplasmic lysates of infected cells by phenol extraction and ethanol precipitation and then suspended in water (45 μ l). Samples (5 μ l) of purified RNAs from cells labeled continuously (●) or pulse-labeled for 1-h periods (■) with [3 H]uridine were assayed for acid-precipitable radioactivity. Portions (20 μ l) of total RNA were digested with micrococcal nuclease, deproteinized by phenol extraction and ethanol precipitation, suspended in 20 μ l of water, and assayed (5 μ l) for acid-precipitable radioactivity (○). RNA from virions isolated from the media of infected cells pulse-labeled with [3 H]uridine was assayed for acid-precipitable radioactivity (□).

assayed for radiolabeled material by precipitation with trichloroacetic acid (4).

Preparation of dsRNA in total RNA. Nuclease-resistant RNA (dsRNA) was prepared by incubating 20- μ l portions of total RNA for 10 min at 20°C in reaction mixtures containing 40 mM Tris hydrochloride (pH 7.5), 0.12 M NaCl, 1 mM CaCl_2 , and 10 μ g of micrococcal nuclease per ml. Reactions were then adjusted to 2 mM EGTA (ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid) to inhibit digestion. Nuclease-resistant RNA was recovered by phenol extraction and ethanol precipitation, assayed for acid-precipitable radioactivity, and analyzed by electrophoresis on agarose-urea gels.

Electrophoresis. Portions (20 μ l) of total- and nuclease-resistant RNA were mixed with 15 μ l of sample buffer (6 M urea, 20% sucrose, 0.1% bromophenol blue, 2.5 mM citrate buffer [pH 3.0]) and analyzed by electrophoresis on 1.75% agarose gels containing 6 M urea and 25 mM sodium citrate buffer (pH 3.0) (23). Gels were run at 170 V until the bromophenol blue dye front migrated 22 cm. Gels were then processed for fluorography and exposed to Kodak XAR-5 film (20). Intensities of bands in the linear range on fluorographs were quantified by using a 2202 Ultrascan densitometer ($A_{632.8}$; LKB Instruments, Inc.) interfaced with an Apple II computer. ^3H -labeled dsRNA and single-stranded RNA (ssRNA) markers were prepared as described previously (20).

Recovery of RNA from extracellular virus. To isolate extracellular virus, the supernatant from infected-cell monolayers was adjusted to 10% polyethylene glycol and was stirred overnight at 4°C. Virus was then pelleted from the supernatants by centrifugation at $65,000 \times g$ for 30 min in a Ti70.1 rotor (Beckman Instruments, Inc.) at 4°C. Pellets were suspended in NTE buffer (0.1 M NaCl, 1 mM EDTA, 0.01 M Tris) containing 0.5% sodium dodecyl sulfate. Virion

RNA was recovered from the suspensions by phenol extraction and ethanol precipitation.

RESULTS

Levels of plus- and minus-strand RNAs. The steady-state levels of rotavirus plus- and minus-strand RNAs were examined by maintaining rotavirus SA11-infected MA104 cells in [^3H]uridine, beginning at 1 h p.i. Cells were then harvested at 3-h intervals from 3 to 15 h p.i. and at 24 h p.i. Total RNA was purified from the cytoplasm of the infected cells, assayed for acid-precipitable radioactivity (Fig. 1), and analyzed by electrophoresis on agarose gels containing 6 M urea (Fig. 2). This gel system allows resolution of the plus- and minus-strand RNAs of the rotavirus genome segments (20).

Most of the ^3H -labeled RNAs produced in infected cells comigrated with rotavirus genome-length plus- or minus-strand RNAs on agarose-urea gels (Fig. 2). However, small amounts of two host-derived ^3H -labeled RNAs were also made in infected cells (lane m). An RNA of unknown origin was detected on some agarose-urea gels. This RNA migrated directly below the band for 4+ RNA.

Between 3 and 9 h p.i. in rotavirus-infected cells, the steady-state levels of total viral RNA (Fig. 1) and both plus- and minus-strand RNAs increased severalfold (Fig. 2). At 9 to 12 h p.i., the levels of viral plus- and minus-strand RNAs were maximal. By continuous labeling, viral plus- and minus-strand RNAs were first readily apparent at 6 h p.i. (pulse-labeling studies presented below show that viral RNA synthesis begins by 3 h p.i.).

As determined by densitometry, the ratio of total plus- to minus-strand RNAs varied between segments in the infected cell. At 12 h p.i., the ratio of plus- to minus-strand RNAs was approximately 1:1 for segment 1, 2:1 for segment 4, 3:1 for segment 5, and 3:1 for segment 6. This variability probably reflects differences in the level of transcription associated with each genome segment.

To characterize the steady-state levels of dsRNAs during infection, portions of total cytoplasmic RNA recovered from infected cells maintained in [^3H]uridine were treated with micrococcal nuclease to remove ssRNA. The resistant RNAs (dsRNAs) were purified by phenol extraction and ethanol precipitation, assayed for radioactivity (Fig. 1), and analyzed by gel electrophoresis (Fig. 3). Like total viral RNAs, the levels of dsRNAs in infected cells increased significantly between 3 and 9 h p.i. and were maximal at 9 to 12 h p.i. The ratio of nuclease-resistant ^3H -labeled minus-strand RNAs remained constant throughout infection, suggesting their coordinate synthesis.

Levels of extracellular dsRNAs. As an indication of the levels of extracellular virion dsRNAs during infection, virus particles were purified at 3 to 15 h p.i. from the supernatant of infected cells maintained in [^3H]uridine. The levels of ^3H -labeled RNAs in virus particles recovered from the media are shown in Fig. 1. A fluorograph of the virion RNAs resolved on an agarose-urea gel is presented in Fig. 4. Radiolabeled plus- and minus-strand RNAs were first detected in extracellular virions at 6 h p.i. and then increased gradually in concentration until 12 h p.i. Afterwards, the rate of accumulation of ^3H -labeled plus- and minus-strand virion RNAs increased more rapidly. The levels of extracellular virion RNAs continued to increase through 24 h p.i. (data not shown).

Synthesis of plus- and minus-strand RNAs. To compare the levels of plus- and minus-strand RNAs synthesis during virus replication, cells were pulse-labeled for 1 h with

[³H]uridine at 3-h intervals between 3 and 15 h p.i. Cytoplasmic RNAs were recovered, assayed for radioactivity (Fig. 1), and examined by electrophoresis on an agarose-urea gel (Fig. 5). The onset of plus- and minus-strand RNA synthesis was detected in infected cells by 3 h p.i. Total RNA synthesis increased significantly between 3 and 9 h p.i., with maximal levels of synthesis occurring at 9 to 12 h p.i. (Fig. 1). At all times examined, transcription and RNA replication were occurring concurrently in infected cells.

Densitometric analysis of fluorographs prepared from gels of the pulse-labeled RNAs indicated that the ratio of plus- to minus-strand RNA synthesis for each segment changed during infection (Table 1). At 3 to 6 h p.i., the ratio of plus- to minus-strand RNA synthesis was approximately 1:1. By 9 h p.i., the ratio was approximately 4:1 to 8:1 and remained relatively constant through 15 h p.i. Although the level of plus-strand RNA synthesis increased after 6 h p.i., the level of minus-strand RNA synthesis remained nearly constant. The synthesis of plus-strand RNA was maximal by 12 h p.i. In some experiments, the ratio of plus- to minus-strand RNA synthesis was greater than 1:1 by 6 h p.i. The source of variability between experiments is not known.

Additional evidence indicating that the ratio of plus- to minus-strand RNA synthesis changes during infection was obtained by pulse-labeling cells, beginning at 3 and 9 h p.i. Cells were then harvested at 1-h intervals for several hours

FIG. 2. Steady-state levels of viral plus- and minus-strand RNAs in infected cells. Total RNA was recovered from cytoplasmic lysates of infected cells maintained continuously in [³H]uridine until harvest. Purified RNAs were analyzed by electrophoresis on a 1.75% agarose gel containing 6 M urea and by fluorography. The positions of the plus- and minus-strand RNAs were determined by electrophoresis of virion-derived dsRNA and are labeled (lane ds) (20). RNAs were recovered from mock-infected cells harvested at 9 h p.i. (lane m), and total viral RNAs were recovered from cells harvested at 3, 6, 9, 12, 15, and 24 h p.i.

FIG. 3. Steady-state levels of viral dsRNAs. Samples (20 μ l) of total RNA recovered from the cells continuously labeled with [³H]uridine were treated with micrococcal nuclease to remove ssRNAs. The nuclease-resistant material was deproteinized and analyzed by electrophoresis on an agarose-urea gel. Nuclease-resistant RNAs were prepared from cells harvested at 3, 6, 9, 12, and 15 h p.i. The positions of viral plus- and minus-strand RNAs are indicated at the right.

after the pulse. The cytoplasmic RNAs were recovered and analyzed by gel electrophoresis (Fig. 6). The ratio of plus- to minus-strand RNA synthesis early in infection (6 to 8 h p.i.) remained near 1:1. The ratio late in infection was approximately 5:1. These data indicate that the level of RNA replication relative to transcription is much higher early in infection than late in infection. In addition, these data show that no direct correlation exists between the level of plus-strand RNA synthesis in the infected cell and the level of RNA replication.

Synthesis of dsRNA. The synthesis of SA11 dsRNA during infection was investigated by purifying cytoplasmic RNA from infected cells that had been pulse-labeled for 1 h with [³H]uridine. The RNA was then treated with micrococcal nuclease to remove single-stranded molecules. Afterward, the nuclease-resistant material (dsRNA) was analyzed by gel electrophoresis (Fig. 7). From 6 to 15 h p.i., nuclease-resistant ³H-labeled plus- and minus-strand RNAs representing all 11 genome segments were detected. The presence of the nuclease-resistant plus-strand RNAs demonstrated that some plus-strand RNAs that were made during the 1-h pulse-labeling were replicated, thus producing dsRNAs. The fact that nuclease-resistant, labeled plus-strand RNAs were found throughout infection indicates that plus-strand RNAs made at all times p.i. can serve as templates for RNA replication.

The quantities of ³H-labeled minus-strand RNAs made during 1-h pulse-labeling that were nuclease-resistant ex-

FIG. 4. Levels of extracellular virion dsRNAs. Virions were recovered from the media of cells maintained continuously in the presence of [^3H]uridine. Virion RNA was purified by phenol extraction and ethanol precipitation and was analyzed by electrophoresis on an agarose-urea gel and fluorography. ^3H -labeled dsRNA (lane ds) and ssRNA (lane ss) markers are shown. The prominent, unidentified bands in lane ss result from reticulocyte lysate in reactions (20). Virion RNA was isolated from the media at 3, 6, 9, 12 and 15 h p.i.

ceeded the corresponding quantities of plus-strand RNAs throughout infection (e.g., Fig. 7, segments 1, 4, 5, and 6). The fact that the amounts of newly synthesized plus- and minus-strand RNAs of each segment were not equal showed that the templates for some of the nuclease-resistant minus-strand RNAs made during the 1-h pulse-labeling were unlabeled plus-strand RNAs. Thus, both newly synthesized and preexisting (>1-h-old) plus-strand RNAs can serve as templates for rotavirus RNA replication. Late in infection

TABLE 1. Ratio of plus- to minus-strand RNA synthesis in rotavirus-infected cells

Time ^a (h p.i.)	Ratio ^b (plus-strand RNA/minus-strand RNA) in segment(s):				
	1	2 and 3	4	5	6
6	0.5	0.5	0.5	1.5	1.6
9	4.3	6.1	5.7	8.2	4.7

^a Cells were harvested at times indicated immediately after pulse-labeling for 1 h.

^b Ratio of intensities of bands on fluorographs produced from an agarose-urea gel of RNAs recovered from cells pulse-labeled with [^3H]uridine. Intensities were determined for bands in the linear range with a laser densitometer. Intensities of RNAs of segments 2 and 3 were considered together, since bands representing 2+ and 3+ cannot be resolved on agarose-urea gels.

FIG. 5. Synthesis of viral plus- and minus-strand RNAs. Cells were pulse-labeled with [^3H]uridine for 1-h periods during infection. After pulse-labeling, cells were harvested and total RNA was recovered from cytoplasmic lysates. ^3H -labeled RNAs were analyzed by electrophoresis on an agarose-urea gel and fluorography. Marker ssRNAs (lane ss), marker dsRNAs (lane ds), and RNAs from cells harvested at 3, 6, 9, 12 and 15 h p.i. are shown.

(12 h p.i.; Fig. 7), however, the amount of newly synthesized plus-strand RNA used as a template for replication was much less than that used at early times (6 h p.i.). This difference may stem from an increase in the ratio of preexisting to newly synthesized plus-strand RNAs during infection. Densitometric analysis indicated that at late times in infection preexisting plus-strand RNAs acted as templates for the synthesis of 80 to 90% (segments 1, 2, and 3 together and segment 4) of the minus-strand RNAs that were made during pulse-labeling.

Rate of release of newly synthesized dsRNA from cells. To investigate the length of time between the synthesis of dsRNA and its release from the cell in virions, infected cells were pulse-labeled with [^3H]uridine for 1-h periods at 2 to 15 h p.i. Afterwards, virus was recovered from the media overlaying the cells. RNAs were isolated from the virions and analyzed by gel electrophoresis. From 6 to 15 h p.i., ^3H -labeled minus-strand RNAs were detected in virions produced by pulse-labeled cells (Fig. 8). Thus, less than 1 h was required between the synthesis of SA11 dsRNAs, i.e., minus-strand RNA synthesis, and its release from the cell within a virion. The lack of ^3H -labeled plus-strand RNAs in virions indicated that most of the plus-strand RNAs used as templates for RNA replication were not synthesized during the pulse-labeling (Fig. 8). Instead, virion dsRNAs were derived primarily by relication of preexisting plus-strand RNAs during the 1-h pulse-labeling.

DISCUSSION

We have made a comprehensive study of the synthesis of viral plus-strand RNAs (transcription) and minus-strand RNAs (replication) in cells infected with simian rotavirus SA11. Rotavirus RNAs were examined by a gel electrophoresis system that allows resolution of viral plus- and minus-strand RNAs (20). In agreement with previous studies (7, 9, 16), viral transcription and RNA replication in our studies began by 3 h p.i. Our data showed that after 3 h p.i., the level of transcription increased until 9 to 12 h p.i., at which time the synthesis of plus-strand RNAs was maximal. In contrast, RNA replication in infected cells reached maximal levels earlier in infection (6 to 9 h p.i.). The delay in obtaining maximal plus-strand RNA synthesis may be due to a requirement for the accumulation of stoichiometric amounts of a protein (e.g., VP6) necessary for the assembly of transcriptase particles (11).

During early phases of infection (<6 to 9 h p.i.), the levels of plus- and minus-strand RNA synthesis were nearly equivalent. The result of this equivalency is likely a rapid amplification in the number of intracellular dsRNA templates. The capacity of the newly formed templates to then synthesize viral mRNAs probably accounts for the increasing level of viral transcription seen later in infection. Although the level of SA11 plus-strand RNA synthesis increased during 6 to 12 h p.i., the level of RNA replication did not increase correspondingly (Fig. 5), suggesting that the level of RNA replication is regulated by factors other than simply the level of plus-strand RNAs in the infected cell.

The synthesis of rotavirus dsRNA requires plus-strand RNA as a template for the synthesis of minus-strand RNA

FIG. 6. Pulse-labeling of total viral RNAs made early and late in infection. Cells were pulse-labeled with [3 H]uridine at 3 and 9 h p.i. For 6 h after addition of the label, at hourly intervals, cells were harvested, and the RNAs were recovered from the cytoplasm of the infected cells and analyzed by electrophoresis on an agarose-urea gel. Labeled RNAs from cells harvested at 4 to 8 h p.i. (left) or 10 to 15 h p.i. (right) are shown with dsRNA (ds) and ssRNA (ss) markers.

FIG. 7. Synthesis of viral dsRNAs during infection. Cells were pulse-labeled with [3 H]uridine for 1-h periods during infection. Total RNA was isolated from the cytoplasm and treated with micrococcal nuclease. The nuclease-resistant material was analyzed by electrophoresis on an agarose-urea gel and by fluorography. Radiolabeled RNAs of cells harvested at 3 to 15 h p.i. are shown with dsRNA markers (lane ds).

(19). We have found that throughout infection, both newly synthesized and preexisting plus-strand RNAs can serve as templates for the synthesis of minus-strand RNA. Early in infection (6 h p.i.), plus-strand RNA readily acted as a template for the synthesis of dsRNA within 1 h of synthesis. Late in infection, preexisting (>1-h-old) plus-strand RNA primarily served as a template for RNA replication. It is not certain why less newly synthesized plus-strand RNA is used for RNA replication at late times of infection rather than at early times. However, this difference may result from an increase in the size of the pool of plus-strand RNA in the cell during infection. Late in infection, a large pool of preexisting plus-strand RNA may dilute the newly synthesized plus-strand RNA such that relatively little of the new RNA is used in RNA replication. We find no evidence to suggest, as reported for the reoviruses (1), that the synthesis of plus-strand RNA destined to act as a template for the synthesis of minus-strand RNA in rotavirus-infected cells is restricted only to early times of infection. However, it is possible that in the reovirus studies, the assay methods employed were not sufficiently sensitive to detect the use of small amounts of plus-strand RNA produced late in infection as templates for minus-strand RNA synthesis.

Our data indicated that the ratio of synthesis of total plus- to minus-strand RNAs for genome segments 1 to 4 was less than 1 at 5 to 6 h p.i. (Table 1). Thus, the synthesis of minus-strand RNAs during this period seems to exceed the rate at which its template, plus-strand RNA, is synthesized. This excess may be possible if preexisting plus-strand RNA

FIG. 8. Rate of release of dsRNA from infected cells. Virions were isolated from the media of cells pulse-labeled for 1-h periods during infection. Virion RNA was recovered by phenol extraction and ethanol precipitation and was analyzed by agarose-urea gel electrophoresis. A prolonged fluorographic exposure (>3 months) is shown of radiolabeled RNA in medium with cells at 3 to 15 h p.i.

is used as a template for replication during this period. However, since the nucleotide sequences of segments 1 to 4 are unknown, it is also possible that the ratios of synthesis appeared to be less than 1 only because the minus-strand RNAs of these segments are unusually rich in uridine residues.

The presence of extracellular dsRNA by 6 h p.i. in the media with infected cells showed that virions were released from rotavirus-infected cells by this time. Afterwards, virion release increased until it reached a maximum at 15 to 24 h p.i. These results agree with those of previous studies, which showed that SA11-infected cells initially produce PFU prior to 6 h p.i. and produce maximum levels of PFU at 18 to 24 h p.i. (7). Examination of virions released from pulse-labeled cells showed that less than 1 h was required between the synthesis of dsRNA and its release from the cell within virions. However, the nature of the structure, i.e., single- or double-shelled virus particles, in which the dsRNA is released has not been determined.

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