Role of Two Particle Types in Bovine Rotavirus Morphogenesis†

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The involvement of light (L) and dense (D) bovine rotavirus particle types during virus replication has been studied. It was found that infectious parental L virions are uncoated *in vivo* to a particle similar to native D particles. Differences in the rate of synthesis and relative yields of L and D particles in MDBK and MA-104 cells have been detected. Results from pulse-chase labeling experiments indicate that D particles serve as morphogenic precursors to the complete L virion.

Rotaviruses, which cause gastroenteritis in a number of animals (20, 25), are members of the family Reoviridae, which also includes reoviruses and orbiviruses. The rotavirus is icosahedral, with two layers of capsid polypeptides (9-11, 15). The genome is composed of 11 segments of double-stranded (ds) RNA of various sizes with a total molecular weight of 10.8×10^6 (2, 14, 19, 23, 24). It has been shown by several workers that CsCl gradient centrifugation of rotaviruses yields light (L) virions and dense (D) particles (4, 8, 16, 17, 24). The L virion has a buoyant density of 1.36, is infectious, and is composed of 11 dsRNA segments and 7 to 10 structural polypeptides (4, 8, 16, 17, 24). The D particle has a buoyant density of 1.38, lacks infectivity, and is composed of the same 11 dsRNA genome segments but only four to five structural polypeptides (4, 8, 16, 17, 24). The morphogenesis of various rotaviruses has been studied in some detail by using the electron microscope (1, 9, 10, 18). Although both the morphogenesis of rotaviruses and the structural components of L virions and D particles have been studied, events during replication that involve the two particle types are unknown. Much of the difficulty in studying the replication of rotaviruses has been the lack of cell culture-adapted strains of the various viruses. We have recently described a culture procedure using trypsin-treated bovine rotavirus that yields sufficient virus to allow the study of replication (5).

The role of L and D particles in the replication of bovine rotavirus is reported in this paper. We have found that infectious parental L virions are rapidly and efficiently uncoated to a particle similar to native D particles. We have also found that newly formed D particles serve as morphogenic precursors to L virions.

The virus used in this study was the Lincoln isolate of bovine rotavirus which was initially provided by Norden Laboratories, Lincoln, Nebr. The virus was originally adapted to cell culture by Mebus et al. (12) and has been partially characterized in this laboratory (2). Virus culture procedures have been described (5).

To study the fate of parental virus during infection of MDBK cells, cells were infected with purified, ^3H -labeled L rotavirions. Changes in density of the labeled, input L virions during infection were determined by centrifuging cell-associated virus, extracted at various times, in CsCl gradients. L virions were rapidly converted to a particle with a buoyant density identical to D particles (Fig. 1). By 1 h postinfection (p.i.), approximately 50% of the input L virus had been converted to particles with a density of D particles. Preliminary studies on the polypeptide composition of the uncoated particle and D particles purified from infected cell culture fluids indicate that both have very similar if not identical polypeptide compositions (S. Clark, manuscript in preparation).

Studying the *in vitro* uncoating of bovine rotavirus L virions, Cohen (6) and Cohen et al. (7) found that removal of outer shell polypeptides with EDTA converted L virions ($P = 1.36$) to D particles ($P = 1.38$), a conversion that activated the virion-associated RNA polymerase. The EDTA-produced D particles had a polypeptide composition identical to D particles purified from both infected fecal material and cell culture fluids. Since the D particles we produced by *in vivo* uncoating also had a buoyant density and polypeptide composition identical to native D particles and, hence, EDTA-produced D particles, these uncoated particles may be involved in transcription of the viral genome during the infection. Studies are currently under way in this laboratory to further define the quantitative aspects of uncoating, RNA polymerase activity,

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FIG. 1. *In vivo* uncoating of L virions. Confluent monolayers of MDBK cells were infected with [^3H]-uridine-labeled L virions that had been purified by two cycles of isopycnic banding in CsCl. Unabsorbed virus was washed from the cultures after absorption for 1 h. At the indicated times, individual cultures were harvested by homogenizing the cells with Freon 113 (0.26 ml of Freon per ml of culture fluids). The homogenates were centrifuged ($10,000 \times g$, 10 min) to separate the phases, and the supernatant was layered onto preformed CsCl gradients ($P = 1.34$ to 1.39 in 0.14 M NaCl , $0.01\text{ M Tris-hydrochloride}$, 1 mM CaCl_2 , $\text{pH } 7.5$). The gradients were centrifuged at $100,000 \times g$ for 90 min and fractionated by bottom displacement. Portions of each fraction were counted by liquid scintillation spectrophotometry. The arrows indicate the position of [^{14}C]uridine-labeled L and D particles included in each gradient.

and polypeptide composition of the uncoated particle.

It was noticed that, while growing bovine rotavirus in MDBK and MA-104 cells, greatly different proportions of L and D particles are produced. To quantify this difference, progeny virus was labeled with [^3H]uridine during growth for 24 h in both cell lines and analyzed with CsCl gradient centrifugation. Tenfold more L virions

were produced in MDBK cells than in MA-104 cells relative to the amount of D particles produced (Fig. 2). The same ratios of L to D were found when [^3H]uridine- or ^3H amino acid-labeled virus was analyzed and when the gradients were analyzed by extinction at 260 nm.

Synthesis of the two particle types was studied by labeling process virus in MDBK cells with [^3H]uridine and analyzing the amount of L and D particles present at various times p.i., using CsCl gradient centrifugation. The total amount of L and D particles present both intracellularly and extracellularly as a function of time is shown in Fig. 3. The number of L virions present increased between 4 and 10 h p.i., with the maximal rate of synthesis at approximately 6 h p.i. The total number of L virions decreased roughly 10% from 10 to 24 h p.i. In contrast, the maximal rate of synthesis of D particles did not occur until approximately 9 h p.i., a lag of 3 h relative to synthesis of L virions. The amount of D particles present continued to increase slowly from 12 to 24 h p.i.

FIG. 2. Yield of L and D particles in MDBK and MA-104 cells. Both cell lines were infected with an input multiplicity of 10. Virus was grown in Eagle minimum essential medium containing $2\text{ }\mu\text{Ci}$ of [^3H]uridine per ml. The cultures were harvested after 24 h and analyzed as described in the legend to Fig. 1.

L and D particle synthesis was also studied in MA-104 cells. The rate of synthesis of D particles in MA-104 cells was similar to that found in MDBK cells, but the synthesis of L virions in MA-104 cells was quite different (Fig. 4). MA-104 cells produced fourfold less L virions, relative to the amount of D particles, than MDBK cells. By 24 h, 60% of the amount of L virions produced in MA-104 cells was lost, in contrast to a loss of only 10% in MDBK cells. The loss of L virions correlates with a loss of infectious virus when measured at the same time during the infection (not shown). When L and D particle synthesis in cell-free and cell-associated MA-104 cell fractions was analyzed, all of the loss of L virions was found to take place in the cell-associated fraction (not shown), indicating that intracellular L virions are very unstable in MA-104 cells. The nature of degraded L virions produced in MA-104 cells is currently being studied.

The synthesis of L and D particle types during pulse-chase and continuous labeling was then studied. Infected cells were pulse-labeled for 30 min at 4 h p.i., washed twice, and then incubated with minimal essential medium containing 100 μ mol of unlabeled uridine, or the infected cells were labeled continuously from 4 h p.i. The infected cells labeled continuously or with a pulse-chase were harvested 30, 60, and 120 min after the addition of unlabeled uridine to the

FIG. 3. Growth of L and D particles in MDBK cells. Bovine rotavirus was grown in MDBK cells in the presence of [^3H]uridine (2 $\mu\text{Ci/ml}$). At the indicated times, the cultures were harvested and analyzed as described in the legend to Fig. 1.

FIG. 4. Growth of L and D particles in MA-104 cells. The experiment was performed as described in the legend to Fig. 3.

pulse-chase samples. The results of a representative experiment are shown in Table 1. When the infected cells were pulse-chase labeled, there was a net 1.1-fold increase in D particles and a 2.9-fold increase in L virions during the 90-min interval from 30 to 120 min after the pulse of [^3H]uridine. When the cells were labeled continuously, there was a 7-fold increase in D particles and an 11-fold increase in L virions during the same time period. The ratio of the increase of L virions to the increase in D particles during this 90-min interval was 2.6 for pulse-chase-labeled infected cells and 1.5 for continuously labeled cells. These results show that, relative to the increase in L virions under both labeling conditions, the increase in D particles during a pulse-chase label was almost half the increase in D particles during continuous labeling.

If D particles were precursors to L virions during morphogenesis, one would expect to see, with the labeling conditions described above, a transfer of radioactivity from D particles to L virions with time during a pulse-chase label and continued synthesis of D particles with a continuous label. Since mammalian cells have large pools of nucleic acid precursors, we were unable to show a transfer of radioactivity from D to L particles. However, we were able to demonstrate a small (8%) loss of D particle from 60 to 120 min after the pulse and a decreased relative rate of synthesis of D particles during pulse-chase labeling compared with continuous labeling. From these results we conclude that the D particle is a morphogenic precursor to the L virion.

Although only subviral particles with a density similar to native D particles were detected in this study, several subviral particles have been

TABLE 1. *L* and *D* particle synthesis under pulse-chase and continuous labeling conditions^a

Labeling condition	Particle	cpm at the following times (min) after pulse:		
		30	60	120
Pulse-chase	D	2,082	2,646	2,441
	L	3,842	7,442	11,185
Continuous	D	3,912	8,464	27,555
	L	6,270	17,082	71,049

^a Confluent monolayers of MDBK cells were infected with bovine rotavirus at a multiplicity of 10. After absorption, minimal essential medium was added to the infected cultures. At 4 h p.i., minimal essential medium containing 25 μ Ci of [³H]uridine per ml was added to the cultures. After 30 min, half of the cultures were washed and fed with minimal essential medium containing 100 μ M of uridine per ml. At the indicated times after the addition of uridine, continuously labeled and pulse-chase-labeled samples were harvested and analyzed as described in the legend to Fig. 1. Each value indicates total counts per minute of material banding at either $P = 1.36$ (L) or 1.38 (D).

found in studies of the morphogenesis of reovirus (13, 26). These reovirus subviral particles differed from each other in polypeptide composition, in time of appearance during the infection, and in the nature of endogenous RNA polymerase activity (13, 26). In pulse-chase labeling studies, two of the reovirus subviral particles were found to act as precursors to the mature reovirion (26) in a manner somewhat analogous to the results of the present study. We may have failed to detect additional particles because we used buoyant density separation in these experiments, whereas sedimentation was used to study reovirus subviral particles. Additional particles with buoyant densities similar to D particles may have been present during morphogenesis. Band widths of D particles in CsCl, however, were identical whether virus accumulated late in the infection during a continuous label or virus labeled during a short time period early in the infection was analyzed, indicating little if any heterogeneity in the D particle band due to the appearance or disappearance of additional particles at different times during the infection.

Several other differences in the morphogenesis of reovirus and rotavirus have been found. First, reovirus morphogenesis has been found to be associated with microtubules in infected cells (20, 21), an association not found in rotavirus replication (1, 9, 10, 18). Second, several electron microscopic studies have demonstrated that rotavirus particles accumulate in the distended cisternae of the rough endoplasmic reticulum (1, 9, 10, 18), an association not seen in reovirus replication. Third, no subviral precursor parti-

cles have been found to accumulate in wild-type reovirus-infected cells. In the present study (Fig. 3 and 4), the synthesis of D particles continued at the times when L virions were no longer being produced. We also found that the ratio of L to D particles produced during a 60-min pulse of [³H]uridine at various times during the infection decreased as the infection proceeded, particularly after maximum amounts of L had been produced (unpublished data). These results indicate that rotavirus-infected cells lose the ability to produce complete L virions while still able to assemble D particles, which leads to an accumulation of precursor D particles.

The observations described above indicate, at the least, that although reovirus morphogenesis and rotavirus morphogenesis are similar in many respects, several events during replication of the two viruses differ significantly.

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