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The Reovirus Replicative Cycle: Conservation of Parental RNA and Protein*

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Abstract. The fate of parental reovirions in the viral replicative cycle has been analyzed using CsCl density centrifugation. After penetration of L-cells, reovirus is converted from a particle of density 1.39 g/cm^3 to a subviral particle of density 1.41 g/cm^3. This alteration in density is temporally correlated with the hydrolysis of viral coat proteins and is qualitatively similar when particles are labeled in their RNA or protein. Ten hours after infection, when synthesis of progeny virus is underway, the parental RNA and protein are again found at density 1.39 g/cm^3. These data demonstrate conservation of the parental RNA and protein in the subviral particle throughout the replicative cycle.

Reovirus is composed of two concentric icosahedral protein shells; the double-stranded RNA genome of the virus lies within the inner shell.[1,2] Removal of the outer shell of capsomeres *in vitro* converts the virion from a structure 75 nm in diameter, with a ratio of RNA to protein of ~1:6 and density ~1.39 g/cm^3, to a subviral particle (SVP) 45–50 nm in diameter, with a ratio of RNA to protein of ~1:2 (ref. 3) and density ~1.43 g/cm^3 (ref. 4). Recently, an RNA transcriptase, which efficiently transcribes single-stranded RNA copies of the double-stranded RNA template, has been shown to be an integral part of the virion.[4–7] This transcriptase activity becomes manifest *in vitro* only upon removal of the outer capsomeres.

Previous experiments demonstrated the segregation of reovirus into lysosomes soon after penetration of the host cell. Lysosomal hydrolases were shown to digest 40–50% of the viral protein, but to have no effect upon the viral RNA.[8] The experiments described below were designed to investigate the fate of the parental RNA and protein during viral uncoating and gene activation *in vivo*. By using isotopically labeled virus, we have been able to trace the proteins and the RNA in the viral inoculum. Our data demonstrate that the parental virions are efficiently converted from particles of density ~1.39 g/cm^3 to SVPs of density ~1.41 g/cm^3 soon after penetration of the host cell, and that these SVPs catalyze the synthesis of viral RNA.[9]

Materials and Methods. Growth of cells and virus: L-929 cells and reovirus type 3 were grown and the virus was labeled, purified, and assayed as described previously.[8] RNA- and protein-labeled virus was grown in nutrient medium—(minimal Eagle's medium (MEM) plus 10% fetal bovine serum)—to which 5 μCi/ml each of [^{3}H]uridine and [^{3}H]cytidine or 15 μCi/ml each of [^{3}H]leucine, [^{3}H]valine, and [^{3}H]-

phenylalanine (New England Nuclear Corp.) were added respectively. 1 ml of an appropriate dilution of labeled virus was adsorbed to a monolayer of about ~5–6 × 10^6 cells in a 10-cm Petri dish for 2 hr at 4°C.

Preparation of cytoplasmic extracts: At the desired interval after adsorption, the cell monolayer was washed with cold PBS (phosphate-buffered saline)[10] and treated with trypsin (0.5 mg/ml in phosphate-buffered saline) to detach the cells. Nutrient medium was added to inhibit the protease. The cells were isolated, washed once in PBS, resuspended in 1 ml of PBS, and lysed after the addition of 2 ml of TL buffer (0.15 M NH_4Cl–0.01 M Tris pH 7.4–2 mM MgCl–0.05% Triton X-100). The lysate was stirred vigorously with a Vortex mixer and then centrifuged at 1500 rpm to separate nuclei and large debris from the cytoplasmic fraction. With this method approximately 90% of the DNA sedimented in the nuclear pellet whereas over 95% of the RNA-labeled reovirus was recovered in the cytoplasmic fraction.

CsCl gradient centrifugation: The cytoplasmic fraction was brought to an average density of 1.4 g/cm^3 by addition of solid CsCl (Schwarz) and to a final volume of 4.5–5.0 ml with TL buffer, and was centrifuged at 4°C for 15 hr at 37,500 rpm in either an SW50.1 or SW39 rotor in a Beckman L-2 ultracentrifuge. The radioactivity and the densities of the gradient fractions were determined as described.[5,8]

Results. (1) Influence of the cytoplasmic extract upon viral buoyant density: The density of the intact virus was unaffected by exposure to cytoplasmic extracts (Fig. 1), or by adsorption of the virus to cells before sedimentation analysis in CsCl (Fig. 2).

(2) Fate of the parental genome: Previous experiments had shown that coat protein hydrolysis was initiated at 20–30 min after viral penetration of the host cell. Three hours after infection coat protein degradation had reached a

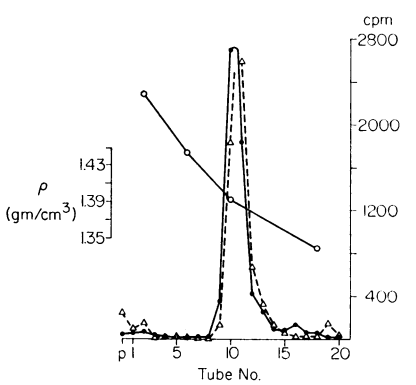

FIG. 1. A cytoplasmic fraction from 10^7 cells was prepared as described, mixed with 10^8 plaque-forming units (PFU) of [^{3}H]RNA-labeled reovirus, and analyzed by CsCl gradient centrifugation (△- - -△). An identical aliquot of labeled virus was analyzed in a separate gradient (●——●) and the results were plotted together for comparison. The density profiles of the two gradients were superimposable and are plotted together (○——○).

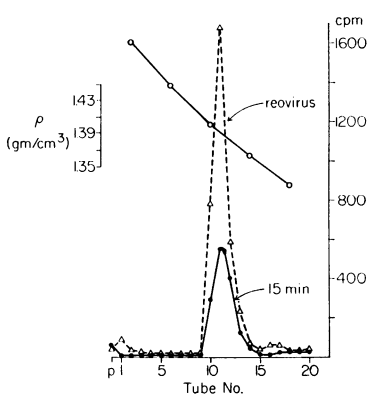

FIG. 2. 2 × 10^8 PFU of [^{3}H]RNA-labeled reovirus was adsorbed to 5 × 10^6 L-cells at 4°C. 15 min after warming to 37°C the virus cell complexes were harvested and a cytoplasmic fraction was prepared. The [^{3}H]RNA-labeled virus in this fraction was analyzed by CsCl gradient centrifugation (●——●). An aliquot of the [^{3}H]RNA-virus was analyzed in a separate gradient in the absence of cytoplasmic extract (△- - -△) and the results were plotted together for comparison, as in Fig. 1.

maximum, but synthesis of progeny virus had not yet begun. At 10–11 hr, progeny virus synthesis was in the mid-to-late exponential phase.[8] Hence, [^{3}H]RNA-labeled virus was adsorbed to cells and cytoplasmic fractions were prepared at 20 min, 3 hr, and 10–11 hr after infection and were centrifuged in CsCl. The resulting gradient fractions were analyzed for radioactivity and density.

At 20 min (Fig. 3*a*), two peaks of radiolabeled RNA, with buoyant densities

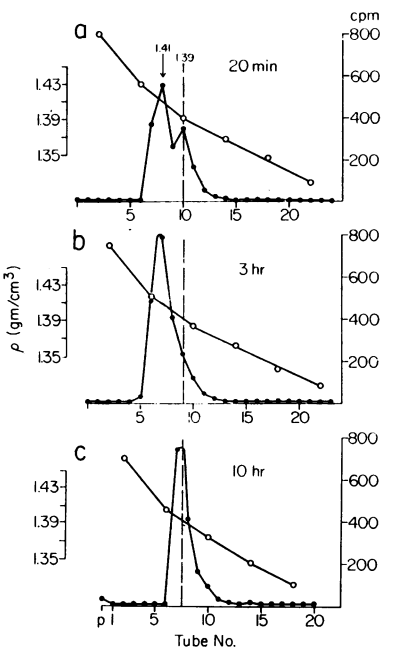

FIG. 3. 2.5 × 10^8 PFU of [^{3}H]RNA-labeled reovirus was adsorbed to 5 × 10^6 L-cells in separate Petri dishes at 4°C. At 20 min, 3 hr, and 10 hr after viral penetration the cells were harvested and cytoplasmic fractions were prepared and analyzed on CsCl gradients.

of 1.39 and 1.41 g/cm^3, were observed. By 3 hr after infection (Fig. 3*b*) the peak at 1.39 g/cm^3 had entirely disappeared, and the bulk of the radioactivity was found in a peak at 1.41 g/cm^3. At 10 hr (Fig. 3*c*) the radioactivity was again recovered in a peak at 1.39 g/cm^3, a density identical to that of the infecting virus. As shown in Table 1, virtually all of the cell-associated radioactivity was recovered in the cytoplasmic fraction, and approximately 90% of the radiolabel in this fraction was recovered in the CsCl gradient fractions. Hence, the radioactivity observed in the gradient fractions represents nearly all the [^{3}H]-RNA present in the cells, and not a minor or selected fraction.

TABLE 1. *Recovery of [^{3}H]RNA-labeled reovirus.*

	3 hr		10 hr	
	Radioactivity (cpm)	Percentage recovered	Radioactivity (cpm)	Percentage recovered
Total homogenate	2440	...	2110	...
Nuclei	60	2	125	6
Cytoplasmic fraction	2400	98	2100	99
Total	2460	100	2225	105
CsCl gradient	2233	93*	1810	86*

* Percentage of radioactivity in the cytoplasmic fraction recovered in the CsCl gradient fractions.

This series of experiments indicated that: (*a*) The virus that had been processed by L-cells was converted from density 1.39 g/cm^3 to 1.41 g/cm^3 3 hr after infection. (*b*) The labeled RNA of the inoculum was fully conserved and appeared at a buoyant density similar to that of parental virus at 10 hr after infection. (*c*) The parental RNA remained in a nucleoprotein complex throughout the replicative cycle, since a density of 1.41 g/cm^3 is significantly lower than the density of viral RNA (1.61 g/cm^3),[11] and higher than the density of viral proteins (1.28 g/cm^3).[12] To test these conclusions by an independent means we examined the fate of protein-labeled virus.

(3) Fate of the parental coat proteins: Previous experiments[8] indicated that coat protein hydrolysis was independent of the multiplicity of infection and that 40–50% of the viral protein was degraded. As shown in Fig. 4, hydrolysis of

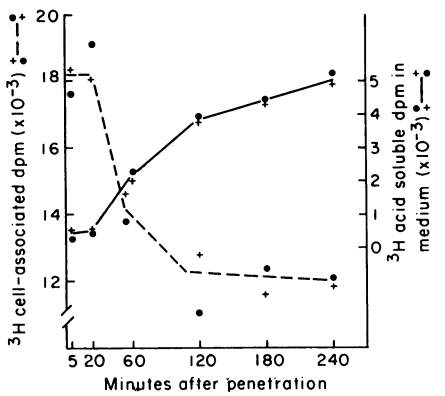

FIG. 4. Hydrolysis of reovirus coat proteins. [^{3}H]protein-labeled reovirus–L-cell complexes were formed in suspension at 4°C at a multiplicity of 100 PFU/cell. After adsorption the cells were washed and placed in warm medium at a density of 8 × 10^5 cells/ml. Half the cells received 20 μg/ml cycloheximide. At appropriate times 3.2 × 10^6 cells from both the cycloheximide-treated (+) and control cultures (•) were harvested by centrifugation. The radioactivity in the acid-insoluble precipitates (cells) and acid-soluble supernatants (medium) was assayed.

coat protein began approximately 20 min after infection and was nearly completed 2 hr after infection. The degree and rate of degradation were similar whether the cells were infected in the presence or absence of cycloheximide. The loss of acid-insoluble radioactivity from the cells was matched by the accumulation of acid-soluble radioactivity in the medium. This experiment, together with the data previously reported,[8] showed that most of the radiolabel lost from the parental coat protein(s) appeared in an acid-soluble form in the medium; hence, little was reincorporated into cellular or viral proteins. Moreover, the hydrolysis of coat protein(s) in the presence of cycloheximide demonstrated directly that cellular protein synthesis was not required for viral uncoating.

(4) Conservation of viral core proteins: To determine whether coat protein hydrolysis was related to the conversion of RNA-labeled virus to a higher density (1.41 g/cm^3), a series of experiments similar to that described in Figure 3 was performed with [^{3}H]protein-labeled reovirus. This virus was adsorbed to cells at 4°C; cytoplasmic fractions were prepared 3 and 11 hr after penetration and analyzed as in the experiments described in Fig. 3.

In agreement with the results obtained using RNA-labeled virus (Fig. 2), the radiolabeled viral proteins exhibited a density of 1.39 g/cm^3 15 min after

infection; by 3 hr the remaining protein label had shifted to a density of 1.41 g/cm³ (Fig. 5*a*). Ten hours after infection (Fig. 5*b*) parental label was again found at density 1.39 g/cm³, a result similar to that described in Fig. 3*c* for RNA-labeled virus.

These results indicated that the protein-labeled virus behaved in CsCl gradients in a manner qualitatively similar to RNA-labeled virus. They further suggested that removal of coat protein(s) from the virus was the factor responsible for the shift in density from 1.39 to 1.41 g/cm³ since coat protein hydrolysis is initiated 20 min after penetration and is nearly completed by 2 hr (Fig. 4). It seemed likely that the return shift from density 1.41 to 1.39 g/cm³, observed at 10–11 hr, resulted from rewrapping of the parental nucleoprotein complex (SVP) with newly synthesized viral proteins.

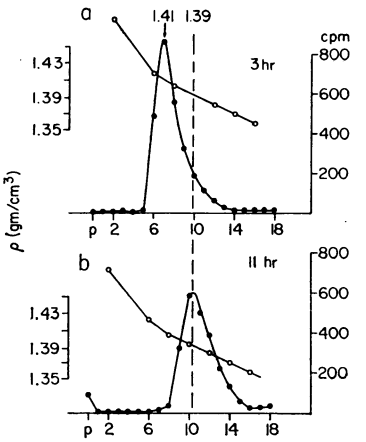

FIG. 5. [³H]protein-labeled reovirus was adsorbed to L-cells at a multiplicity of 1000 PFU/cell. 3 and 11 hr after viral penetration the cells were harvested and cytoplasmic fractions were prepared and analyzed on CsCl gradients. 20-μl aliquots of the gradient fractions were assayed for radioactivity directly.

(5) Effect of cycloheximide on the recoating of the parental nucleoprotein complex: The experiments presented in Fig. 4 indicated that coat protein hydrolysis was unaffected by cycloheximide. Synthesis of new viral proteins, however, was

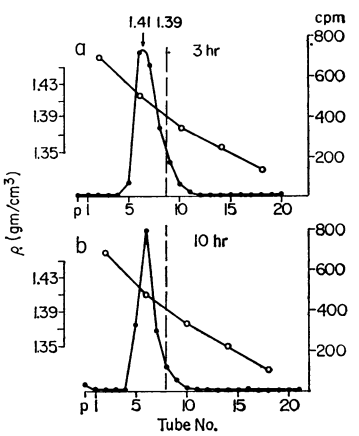

FIG. 6. 2.5 × 10⁸ PFU of [³H]RNA-labeled reovirus was adsorbed to 5 × 10⁶ L-cells in separate Petri dishes at 4°C. After adsorption the cells were overlaid with medium containing cycloheximide (20 μg/ml) and incubated at 37°C. At 3 and 10 hr the cells were harvested, fractionated, and analyzed as in Fig. 3.

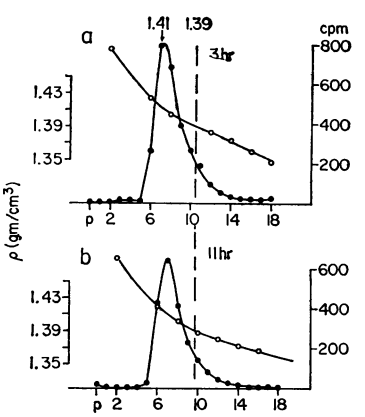

FIG. 7. [³H]protein-labeled reovirus–L-cell complexes were formed at a multiplicity of 1000 PFU/cell. After adsorption the cells were overlaid with medium containing cycloheximide and incubated at 37°C. At 3 and 11 hr the cells were harvested, fractionated, and analyzed as in Fig. 5.

completely inhibited at the concentration of cycloheximide employed.[13] Therefore we used this antibiotic to analyze further the density shifts described for protein- and RNA-labeled virus.

Cells were infected with [^{3}H]protein- or [^{3}H]RNA-labeled reovirus in the presence of cycloheximide, and 3 and 10–11 hr after penetration the cytoplasmic fractions were analyzed as described in Figs. 3 and 5. The results were qualitatively the same for both the protein- (Fig. 6) and RNA-labeled viruses (Fig. 7). Both RNA and protein-labeled virions were converted to particles of density 1.41 g/cm^3 3 hr after infection. 10–11 hr after infection in the presence of cycloheximide, however, both RNA and protein labels remained at density 1.41 g/cm^3, a result directly opposite to that obtained in the absence of this antibiotic (Figs. 3*c* and 5*b*).

These data confirm that uptake and uncoating of virus are independent of protein synthesis[8] and indicate that newly synthesized proteins, presumably specified by the viral genome, are required to return parental RNA and protein to density 1.39 g/cm^3.

Discussion. Using equilibrium density centrifugation we have analyzed the fate of the reovirus inoculum at representative points in the replicative cycle. The purified RNA- and protein-labeled virus preparations used in these experiments had a buoyant density in CsCl of 1.39 g/cm^3.

Hydrolysis of viral coat protein was initiated approximately 20 min after penetration of the host cell. Beginning at this time the parental virions exhibited an increase in buoyant density, shifting from their initial density of 1.39 g/cm^3 to 1.41 g/cm^3. Inhibition of cellular protein synthesis did not alter either the rate or extent of coat protein hydrolysis (Fig. 4). Three hours after infection coat protein hydrolysis was nearly completed, and the majority of the labeled parental viral RNA and protein exhibited a density of 1.41 g/cm^3.

This sequence of events is reminiscent of the enzymatic uncoating and gene activation described for reovirus *in vitro*. Removal of the outer viral capsomeres *in vitro* converts the intact particle to an SVP of density 1.43 g/cm^3 and activates the RNA transcriptase latent within the virion.[4,5,7] A similar activation of the viral transcriptase *in vivo* was suggested by the experiments of Watanabe *et al.*[14] and has been demonstrated directly by recovery of parental SVPs of density 1.41 g/cm^3 from cells infected for 3–4 hr in the presence of cycloheximide. After uncoating *in vivo*, these SVPs catalyze the polymerization of radiolabeled nucleotides into acid insoluble products *in vitro*.[9] Moreover, acrylamide gel analyses of the radiolabeled proteins in subviral particles of density 1.41 g/cm^3 demonstrate a pattern similar to that described by Smith *et al*,[15] for SVPs that exhibited RNA transcriptase activity.[9]

Ten hours after infection, when progeny virus synthesis was in mid-to-late exponential phase, parental RNA and protein was again found at density 1.39 g/cm^3. Previous experiments[8] and those in progress have shown that parental RNA and protein are conserved in a macromolecular form, and not merely degraded and reutilized. Hence the decrease in density of the parental RNA and protein suggests that SVPs are recoated with newly synthesized capsomeres. Support for this hypothesis and confirmation of the requirement for virus-

specific proteins were obtained in experiments in which protein synthesis was blocked with cycloheximide (Figs. 6 and 7). Under these circumstances SVPs of density 1.41 g/cm^3 did not exhibit a reverse shift in density 10–11 hr after infection. The mechanism by which SVPs are converted to particles of lower density, however, remains to be elucidated.

The density shifts described for reovirus argue strongly for conservation of both RNA and protein in a nucleoprotein complex (SVP) since the densities of these components taken separately diverge markedly from the 1.39 and 1.41 g/cm^3 densities observed. Moreover, the quantitative recovery of RNA-labeled particles of buoyant density 1.41 g/cm^3 from cells incubated for 10 hr after infection in the presence of cycloheximide demonstrates the resistance of the SVP to further proteolytic digestion *in vivo*.

From these data the initial events in reovirus infection may be reconstructed. The outer viral capsomeres are removed by proteolytic enzymes within the lysosomes of the host cell. Conversion of the reovirion to an SVP activates the RNA transcriptase within the particle and allows transcription of single-stranded RNA molecules which hybridize with segments of the double-stranded RNA genome.[4,5,7] These single-stranded RNA molecules are exported from the SVP[5] into the cytoplasm and initiate synthesis of viral proteins.

The participation of the parental genome in events occurring late in the infectious cycle cannot be unambiguously reconstructed from these data. SVPs isolated from infected cells at these later points of time appear to be recoated with newly synthesized viral proteins, but the mechanism of this reassembly has not been determined. The reappearance of the major proportion of the parental genome at a density of 1.39 g/cm^3 is consistent with either a semi- or a fully-conservative mode of synthesis of double-stranded RNA. Experiments are now in progress to distinguish between these possibilities.

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Abbreviation: SVP, subviral particle; PFU, plaque-forming units.

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